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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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TITLE OF THE INVENTION (280 characters max)

PRODUCTION AND PURIFICATION OF RECOMBINANT HUMAN ARYL SULPHATASE A

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ENCLOSED APPLICATION PARTS (check all that apply)

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Respectfully submitted,

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PRODUCTION AND PURIFICATION OF RECOMBINANT HUMAN ARYL SULPHATASE A

FIELD OF INVENTION

The present invention relates to a process for production and purification of recombinant human Arylsulphatase A (rhASA) enzyme and the use of rhASA obtained by this process for preventing or alleviating the symptoms related to Metachromatic leukodystrophy.

BACKGROUND OF THE INVENTION

Myelin metabolism and Metachromatic leukodystrophy

Metachromatic leukodystrophy (MLD) is caused by an autosomal recessive genetic defect in the lysosomal enzyme Arylsulfatase A (ASA), resulting in a progressive breakdown of membranes of the myelin sheath (demyelination) and accumulation of galactosyl sulphatide (cerebroside sulphate) in the white matter of both the central nervous system (CNS) and the peripheral nervous system. In histologic preparations, galactosyl sulphatide forms spherical granular masses that stain metachromatically. Galactosyl sulphatide also accumulates within the kidney, gallbladder, and certain other visceral organs and is excreted in excessive amounts in the urine.

Multiple sulfatase deficiency (MSD) is a rare form of MLD that also includes features of mucopolysaccharidosis (MPS). MSD is characterised by a decreased activity of all known sulfatases. The clinical phenotype of MSD combines features of MLD with that of MPS as a result of the impaired lysosomal catabolism of sulphated glycolipids and glycosaminoglycans.

Galactosyl sulfatide is normally metabolised by the hydrolysis of 3-*O*-sulphate linkage to form galactocerebroside through the combined action of the lysosomal enzyme arylsulfatase A (EC 3.1.6.8) (Austin et al. Biochem J. 1964, 93, 15C-17C) and a sphingolipid activator protein called saposin B. A profound deficiency of arylsulfatase A occurs in all tissues from patients with the late infantile, juvenile, and adult forms of MLD (see below). In the following, the arylsulfatase A protein will be termed "ASA" and the saposin B will be termed "Sap-B". A profound deficiency of ASA occurs in all tissues from patients with MLD.

ASA has been purified from a variety of sources including human liver, placenta, and urine. It is an acidic glucoprotein with a low isoelectric point. Above pH 6.5, the enzyme exists as a monomer with a molecular weight of approximately 100 kDa. ASA undergoes a pH-dependent polymerisation forming a dimer at pH 4.5. In human urine, the enzyme consists of two nonidentical subunits of 63 and 54 kDa. ASA purified from human liver, placenta,

and fibroblasts also consist of two subunits of slightly different sizes varying between 55 and 64 kDa. As in the case of other lysosomal enzymes, ASA is synthesised on membrane-bound ribosomes as a glycosylated precursor. It then passes through the endoplasmic reticulum and Golgi, where its *N*-linked oligosaccharides are processed with the formation of phosphorylated and sulfated oligosaccharide of the complex type (Waheed A et al. Biochim Biophys Acta. 1985, 847, 53-61, Braulke T et al. Biochem Biophys Res Commun. 1987, 143, 178-185). In normal cultured fibroblasts, a precursor polypeptide of 62 kDa is produced, which translocates via mannose-6-phosphate receptor binding (Braulke T et al. J Biol Chem. 1990, 265, 6650-6655) to an acidic prelysosomal endosome (Kelly BM et al. Eur J Cell Biol. 1989, 48, 71-78).

The length (18 amino acids) of the human ASA signal peptide is based on the consensus sequence and a specific processing site for a signal sequence. Hence, from the deduced human ASA cDNA (EMBL GenBank accession numbers J04593 and X521151, see below) the cleavage of the signal peptide should be done in all cells after residue number 18 (Ala), resulting in the mature form of the human ASA. In the following, the mature form of the human ASA will be termed "mASA" and the mature recombinant human ASA will be termed "mrhASA".

Multiple forms of ASA have been demonstrated on electrophoresis and isoelectric focusing of enzyme preparations from human urine, leukocytes, platelets, cultured fibroblasts and liver. Treatment with endoglycosidase H, sialidase, and alkaline phosphatase reduces the molecular size and complexity of the electrophoretic pattern, which suggests that much of the charge heterogeneity of ASA is due to variations in the carbohydrate content of the enzyme.

Clinical manifestations of MLD

The central nervous system consists of the brain and the spinal cord, and can be divided into white and grey matter. The white matter consists of nerve cells, and in MLD the damage occurs primary in the nerve cells. When the nerve cells are damaged, they can no longer conduct nerve impulses to muscles, skin and internal organs.

In cases of MLD, there is a defect in ASA activity affecting myelin metabolism. Lack of this enzyme in patients with MLD leads the degradation of myelin and to dysfunction of the nerve cells. A concomitant accumulation of special types of fat in the nerve cells is also observed in MLD.

Three forms of the disease can be distinguished according to the three forms of the age of onset: Late-infantile, juvenile and adult (after the age of 20 years).

The course of the disease varies in the different types. The type occurring in early childhood is the commonest, progresses most rapidly, and leads to pronounced handicapping and death.

In the infantile form of MLD there are several stages of the disease. The first stage is characterised by slack muscles (hypotonia) of the arms and legs. Walking deteriorates and the child needs support to walk. The picture is often complicated by disturbances of balance (ataxia) and weakened muscle reflexes. In the second stage, about 1-1½ years after the onset, the child can no longer stand, but it can still sit. The previous slack muscles become spastic. The disturbance of balance get worse, and pain in the arms and legs is common observed. The disease progresses to the third stage after additional 3-6 months where the child has increasing paralysis of all four limbs and can no longer sit. The child gradually needs help with everything, vision is impaired, and movements become difficult.

The juvenile type of MLD starts between the ages of five and ten years. The progression is similar to the infantile type, but slower. Emotional lability and impaired vision may be the first symptoms of the disease.

In the adult form of MLD the symptoms arise in the age after 20 years after normal development. The symptoms include cognitive and behavioural abnormalities.

Incidence of MLD

In Norway, about one child with MLD is born every year, i.e. a frequency of about 1:50.000. Similar results have been obtained in northern Sweden where the birth incidence rate for late infantile MLD in this population can be calculated to be about 1 per 40.000. Only one case of juvenile MLD was born in the mentioned region during the same period. This demonstrates that the juvenile form of MLD is much more rare than the infantile form.

Existing diagnosis of MLD

In order to diagnose MLD, examination of spinal fluid, urine, various blood tests, and analysis of the ASA activity can be carried out. Deficiency of ASA activity in material from

patients with MLD (e.g. peripheral leukocytes and cultured skin fibroblasts) can be investigated. Analysis of the urine from patients with MLD can indicate a defect at the level of myelin metabolism but this is a less reliable source for diagnostic assays because the urinary enzyme level is normally highly variable. Excessive amounts of sulpatide excreted in the urine and metachromatic granules in the urinary sediment are observed. Furthermore, normal x-rays and computer tomography (CT) of the head may be carried out.

Prenatal diagnosis appears to be possible by measuring ASA activity in cultured cells from amniotic fluid or chorionic villus cells. Cerebroside sulfate loading of such cells can also be used and is the method of choice if the pseudodeficiency gene is also present in the family.

Existing treatment of MLD

There are relatively few treatment options for MLD. Bone Marrow Transplantation (BMT) has been used in the treatment of more than 20 patients with MLD (for instance Bayever E et al. Lancet 1985, 2, 471-473), and it appears that BMT slows the progression of symptoms, but benefits of the treatment are not seen for several months. In most late infantile patients, symptoms are progressing rapidly by the time of diagnosis, and the risks of the procedure tend to outweigh the possible benefits. In instances in which the diagnosis can be made presymtomatically and a well-matched donor is available, BMT may be a reasonable approach. Moreover, reported results suggest that BMT is efficacious only in MLD patients with high residual activity or when performed in presymptomatic stages in the late infantile form probably because of the rapid progression of the disease.

Cell culture models suggest that cysteine protease inhibitor treatment (von Figura K et al. Am J Hum Genet 1986, 39, 371-382), thiosulfate treatment (Eto Y et al. Biochem Biophys Res Commun 1982,106, 429-434), enzyme replacement (Porter MT Science 1971, 172 (989),1263-1265), and gene replacement therapies (Sangalli A et al. Hum Gene Ther 1998, 9, 2111-2119) could be effective. Several possible gene therapy approaches have been suggested.

In one of these approaches an implanted polymer-encapsulated xenogenic transduced cell line secreting the ASA enzyme is used. This approach has previously been used for the treatment of other neurological disorders such as Amyotrophic Lateral Sclerosis and Parkinson disease. A cathetered devise, containing around 106 genetically modified cells surrounded by a semipermeable membrane, is suggested to be implanted in the ventricular space, providing slow continuous release of ASA directly in cerebral spinal fluid. For this gene transfer technique C2C12 mouse myoblast cells are used (Deglon et al. Hum

Gene Ther 1996, 7, 2135-2146). The semipermeable membrane prevents immunologic rejection of the cells and interposes a physical barrier between cells and host. Moreover, the device and the cells may be retrieved in the event of side effect due to the ASA administration.

In another approach ASA genes are directly delivered into the brain by the use of recombinant adenovirus (Ohashi et al. Acta Paediatr Jpn. 1996, 38, 193-201). It was shown that the recombinant adenovirus (Adex1SRLacZ) was able to transduce the oligodendrocytes very efficiently. Hence, it was concluded that the correction of ASA deficiency by a recombinant adenovirus that potentially could be used to transfer the ARSA gene to the brain, and gene therapy for MLD based on gene transfer of the ARSA gene to mutant cells will be feasible, because the overexpression of ASA in cells does not lead to profound deficiency of other sulfatases or result in a new phenotype.

SUMMARY OF THE INVENTION

In essence, the inventive concept of the present invention is based on the finding that isolation of rhASA from a mammalian cell system, which is cultured in a system allowing for continuous cell propagation, and purification by a series of specific chromatography steps implies a number of important advantages, including an increased expression level and yield of recombinant protein as well as increased purity of the rhASA obtained. Such purified rhASA is suitable for use in pharmaceutical preparations and may be produced in a form that will be able to cross the blood-brain barrier.

Accordingly, the present invention pertains to a process for production of recombinant human arylsulfatase A in a cell culture system, the process comprising:

i) culturing a mammalian cell capable of producing rhASA in liquid medium in a system comprising one or more bio-reactors;

ii) concentrating, purifying and formulating the rhASA by a purification process comprising one or more steps of affinity chromatography.

In a preferred embodiment, the concentration and purification process of ii) comprises the following steps:

I) concentrating rhASA present in the liquid medium by tangential flow filtration;

II) loading the rhASA containing supernatant obtained in step I on an equilibrated chromatography column and eluting one or more fraction(s) containing rhASA;

III) loading the fraction(s) from step II on another equilibrated chromatography column and eluting one or more fraction(s) containing rhASA;

IV) purifying rhASA present in the fraction(s) from step III by tangential flow filtration;

V) polishing the preparation of rhASA from step IV in one or two or more successive steps, each step comprising loading the preparation on an equilibrated chromatography columns and eluting one or more fraction(s) containing rhASA;

VI) passing the fraction(s) from step V through a viral reduction filter;

VII) formulating the fraction(s) from step VI in order to obtain a preparation of rhASA in a suitable formulation buffer;

VIII) optionally filling the formulated preparation of rhASA into a suitable container and freeze-drying the sample.

Other aspects of the invention provides a pharmaceutical composition comprising rhASA, which is efficiently endocytosed via the mannose-6-phosphate receptor pathway *in vivo* as well as a rhASA a medicament and use of a rhASA for the manufacture of a medicament for reducing the galactosyl sulphatide levels within target cells in the peripheral nervous system and/or within the central nervous system in a subject.

A final aspect of the invention provides a method of treating a subject in need thereof, said method comprising administering to said subject a pharmaceutical composition comprising a rhASA and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within said subject.

DETAILED DESCRIPTION OF THE INVENTION

"recombinant" refers to a DNA or polypeptide molecule produced from recombination, especially that produced from two different species by genetic engineering.

"Blood-brain barrier" are endothelial cells that line the brain capillaries.

By the term "catalyst" is herein meant either the relevant enzyme which is substituted as it is, or an enzymatically equivalent part or analogue thereof. One example of an enzymatically equivalent part of the enzyme could be a domain or sub-sequence of the enzyme, which includes the necessary catalytic site to enable the domain or sub-sequence to exert substantially the same enzymatic activity as the full-length enzyme.

An example of an enzymatically equivalent analogue of the enzyme could be a fusion protein which includes the catalytic site of the enzyme in a functional form, but it can also be a homologous variant of the enzyme derived from another species. Also, completely synthetic molecules which mimic the specific enzymatic activity of the relevant enzyme, would also constitute "enzymatic equivalent analogues".

A "contaminant" is a material that is different from the desired polypeptide product. The contaminant may be a variant of the desired polypeptide (e. g. a deamidated variant or an amino-aspartate variant of the desired polypeptide) or another polypeptide, nucleic acid, endotoxin etc.

A "disorder" is any condition that would benefit from treatment with the polypeptide purified as described herein.

To "elute" a molecule (e. g. polypeptide or contaminant) from an ion exchange material is meant to remove the molecule therefrom by altering the ionic strength of the buffer surrounding the ion exchange material such that the buffer competes with the molecule for the charged sites on the ion exchange material.

By "purifying" a polypeptide from a composition comprising the polypeptide and one or more contaminants is meant increasing the degree of purity of the polypeptide in the composition by removing (completely or partially) at least one contaminant from the composition. A "purification step" may be part of an overall purification process resulting in a composition comprising at least about 20% by weight of the polypeptide of interest, based on total weight of the composition, preferably at least about 30% by weight.

By the term "target cell" is herein meant a cell or group of cells (tissue) to which the enzymes should be delivered.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

By "washing" the ion exchange material is meant passing an appropriate buffer through or over the ion exchange material.

The present invention relates to an additional strategy for the treatment of MLD according to which recombinant human Aryl Sulphatase A (rhASA) is administered directly over the cellular membranes and/or the blood-brain barrier in order to reach the relevant target

cells. While basically all cells in the brain are deficient of the ASA, cell types of particular interest are oligodendrocytes or oligodendroglia that are responsible for myelination of neurons within the central nervous system and neuronal cells. Schwann cells, which are responsible for myelination of the peripheral nerve system (PNF), are one of the main target cells outside the central nervous system (BBB).

A number of different delivery techniques may be applied to the rhASA enzyme in order to facilitate its transport across the BBB and/or cellular membranes. Examples of such techniques are briefly described in the following paragraphs:

1) Use of mannose-6-phosphate tags produced as post-translational modifications by a combined action of phosphotransferases and phosphoglycosidases in the Golgi apparatus when the rhASA is expressed in a mammalian cell system. The tagged version of the enzyme will have the capacity to cross the cellular membrane via mannose-6-phosphate receptor uptake.

2) Peptides and proteins as vehicles for passage of rhASA to the target cells by passage over cell membranes and/or the BBB:
A number of earlier studies in animals have shown that certain proteins and/or peptides may act as vehicles for passage of BBB. For instance proteins modified by the insulin fragment (Fukuta et al. Phaomacol Res 11: 1681-1688) or antibodies to the transferrin receptor (Friden et al. Proc Natl Acad Sci USA 88: 4771-4775) can pass the blood-brain barrier. Also proteins modified by coupling to polyamines (Poduslo and Curran. J Neurochem 66: 1599-1606) have been reported to pass the blood-brain barrier.

Of particular relevance to the present invention are membrane-disrupting or protein-transducing domains, where the focus has been on short peptides 10-30 residues in length. When covalently attached to protein molecules these peptides can transport the molecule across the blood-brain barrier and also across cellular membranes in general (Schwarze et al.,Trends Cell Biol. 2000; 10(7): 290-295; Lindgren et al., Trends Pharmacol. Sci. 2000; 21(3): 99-103). A modified rhASA molecule containing such peptide sequences can be produced by expression techniques. The protein transduction process is not cell type specific and the mechanism by which it occurs is not fully elucidated, however, it is believed that it takes place by some sort of membrane perturbation and penetration process that is receptor independent. A partially unfolded state of the molecule may facilitate the process but is not essential.

Protein transducing domains are generally derived from viruses or other non- human protein molecules (and have the potential to be immunogenic). Examples of such domains include:

The 11 residue basic peptide from the HIV TAT protein –YGRKKRRQRRR (Schwarze et al.,Trends Cell Biol. 2000; 10(7): 290-295). This peptide binds to extracellular matrix-associated heparan sulfate proteoglycans (HSPGs) and transports a wide variety of large and small molecules across cellular membranes. The initial entry may be vesicular and the transduced molecule comes back out of the cell when the outside concentration decreases. The peptide can be present anywhere in the molecule as long as it is exposed, even in the reverse order of amino acid residues. All humans have low titer innate antibodies to this basic domain of TAT that are of the IgM isotype (Schwarze et al.,Trends Cell Biol. 2000; 10(7): 290-295).

A synthetic version of TAT –YARAAARQARA that confers more alpha-helicity and amphipathic nature to the sequence (Ho et al., Cancer Res. 2001; 61(2):474-477). This peptide is considerably more efficient than TAT and it also has documented effects in vivo. The peptide has no classical nuclear localization signal present, as is the case with the natural TAT sequence and it presents a different immunologic epitope.

A synthetic leader peptide composed of poly –R or a mixture of basic –R and –K residues in combination with other amino acids.

Peptides based on hydrophobic signal sequence moieties from either beta-3 integrin or Kaposi's sarcoma FGF (Dunican et al. Biopolymers 2001; 60(1): 45-60). These are termed membrane permeable sequences and are hydrophobic rather than basic sequences. They are derived from human proteins so their immunogenic potential may be low.

Other tags may have the capacity to direct the enzyme into the relevant target cells by carrier mediated transport. These tags may be a peptide or protein or the functional part of a peptide or protein which has affinity for a specific receptor. Examples of such receptors could be the nerve growth factor (NGF) or brain derived neurotropic factor (BDNF) receptors.

One way of ensuring a more efficient transport of proteins across the BBB is to use specific transport systems. An example of such a system is the transferrin receptor which normal functions to transport transferrin and melanotransferrin across the BBB (Rothenberger et al., Brain Res. 1996, 712, 117-21; Demeule et al., J Neurochem 2002, 83, 924-33). When

attached to the rhASA a full length or synthetic protein or peptide with affinity for the receptor will "pull" the modified rhASA over the blood-brain barrier. An alternative approach is the use of receptor mediated transfer of specific cytokines over the blood-brain barrier exemplified by the transport of TNF-alpha by p55 and p75 receptor (Pan et al., Exp Neurol. 2002 Apr;174(2):193-200; Pan et al., Arch Physiol Biochem. 2001 Oct;109(4):350-353).

3) Toxins as vehicles for passage rhASA to the target cells by passage over cell membranes and/or the BBB:

Different bacteria, plants and animals produce toxins. Toxins have many different targets such as the gut (enterotoxins), nerves or synapses (neurotoxins). Toxins can traverse cell membranes via receptor mediated processes and the embodiment of the present invention is to use toxins as vehicles to passage rhASA to the target cells over cellular membranes and/or the BBB. Use of toxins for delivery of therapeutics in the treatment of Metachromatic Leukodystrophy (MLD) is of particular relevance since preferred target cells of toxins are cells in the central nervous system and/or the peripheral nervous system. Of practical considerations and for safety reasons only the amino acid peptide pertaining to the translocation over cellular membranes and/or the BBB of the toxin is used.

Diphtheria Toxin (DT), from the *Corynebacterium Diptheriae* is a good example of a toxin which may be used as a vehicle. Bacterial toxins exhibit a wide range of toxicities and they fall into groups by structure and function. The toxin binds to a target cell and enters the cell via a receptor, and is reduced to separate fragments. The processed toxin can be divided into the following 3 domains: The catalytic domain (C), the receptor domain (R), and the translocation domain (T).

An embodiment the present invention relates to a method for production and purification of recombinant human rhASA or an enzymatic equivalent part or analogue thereof, which can be used in the prevention or treatment of MLD and/or the symptoms related to this disorder. The success of this strategy, however, is highly dependent on the availability of preparations of rhASA that are of high purity and uniform quality. It is therefore also within the scope of the invention to provide a quality of rhASA which can act as a catalyst in the intracellular metabolism of galactosyl sulfatide to galactocerebroside and thereby substitute for the deficient ASA, which is one of the characteristics of MLD. In a further perspective, it is within the scope of the invention to provide a recombinant form of arylsulphatase A, which is capable of crossing the blood brain barrier and also a form of rhASA, which possesses specific tags for entry into target cells within the brain. A preferred embodiment of the invention, however, is the production and purification of rhASA with a pattern of mannose-6-phosphate tags that allows the enzyme to enter its

target cells by mannose-6-phosphate receptor mediated entry. In particular, it is an object of the present invention to provide a rhASA which is eficiently endocytosed in vivo via the mannose-6-phosphate pathway.

Mature human ASA has three putative N-glycosylation sites i.e. Asn158, Asn184, and Asn 350, which can form the mannose-6-P tag. Asn158, Asn184, and Asn350 are referred to the precursor ASA which has an 18 residue signal peptide. In the mature ASA the mentioned asparagine residues are Asn140, Asn166, and Asn332, respectively. Only two of the N-glycosylation sites (Asn140 and Asn332) undergoes phosphorylation and can acquire the correct mannose-6-P tag and the mannose-6-P synthesis at these two sites via two distinct enzymatic steps.

Suitable host cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodopterafrugiperda (caterpillar), Aedes aegypti (mosquito), Aedes albopictus (mosquito), Drosophila melanogaster (fruitfly), and Bombyx mori have been identified. A variety of viral strains for transfection are publicly available, e. g., the L-1 variant of Autographa californica NPV and the Bm-5 strain of Bombyx mori NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of Spodopterafrugiperda cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CVI line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture); baby hamster kidney cells (BHK); Chinese hamster ovary cells/-DHFR (CHO); mouse Sertoli cells (TM4); monkey kidney cells (CV I); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK); buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562); TRI cells; MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

The present invention provides a process for production of rhASA in a continuous cell culture system, the process comprising:

i) culturing a mammalian cell capable of producing rhASA in liquid medium in a system comprising one or more bio-reactors;

ii) concentrating, purifying and formulating the rhASA by a purification process comprising one or more steps of affinity chromatography.

More specifically, the method comprises the propagation of a mammalian cell line capable of synthesising rhASA in a culture system, which allows for continuous cell propagation and the subsequent extraction and purification of the resulting rhASA in a series of chromatography steps. When presented in a brief outline, the process for production and purification of rhASA may comprise one or more of the following general steps:

A. Culturing of mammalian cells capable of producing rhASA in a cell culture system allowing for continuous cell propagation.

B. Concentration of rhASA from the supernatant and purification of rhASA by a series of chromatography steps wherein the proteins are separated according to their net charge supplemented by filtration procedures based on separation of the proteins according to size.

C. Formulation, filling, and freeze-drying.

of rhASA in a cell culture system allowing the propagation of cell cultures over extended periods of time.

It is preferred that rhASA is produced in mammalian cell or cell line and that said mammalian cell or cell line produces a glycoform of rhASA, which are efficiently endocytosed *in vivo* via the mannose-6-phosphate receptor pathway. Specifically, the preferred glycoform of rhASA comprises an amount of exposed mannose-6-phosphate, which allows efficient endocytosis of rhASA *in vivo* via the mannose-6-phosphate pathway.

It has previously been contemplated that expression rhASA in either CHO, COS and BHK cells ensures correct mannose-6-phosphate tagging on the molecule, which in turn ensures efficient receptor mediated uptake (Stein et al. J Biol Chem.1989, 264, 1252-1259). While this may be true for endocytosis in vitro, the present inventors have observed a markedly increased in vivo endocytosis of rhASA produced in CHO cells as compared to the endocytosis in vivo of rhASA produced in BHK and COS cells. The efficient endocytosis of the enzyme is a prerequisite for obtaining the desired correction of the sulfatide levels in the peripheral nervous system and in visceral organs of the body. Therefore, it is preferred wherein at least one of the produced glycoforms of rhASA is similar to a glycoform produced in CHO cells.

It is equally preferred that the mammalian cells used in the process according to the invention are of human or primate origin. In a currently most preferred embodiment, the mammalian cells are CHO cells and it is further preferred that these cells are are CHO-DG44 cells. In another preferred embodiment a human cell line is used.

In a preferred embodiment of the present invention the cells used for roduction of rhASA comprise a nucleic acid sequence which encodes an amino acid sequence according to SEQ ID NO: 2. In a further preferred embodiment, the rhASA is encoded by SEQ ID NO: 1.

In the process according to the invention, protein purification is simplified since in the system described, rhASA is secreted into the medium.

The cell culture system is based on one or more conventional bioreactors, which are connected to a source of fresh medium and to a system in which the cells can be harvested and the medium collected. A part of this system may be a cell retention device. Preferably these different parts of the system are interconnected in a way so that fresh medium can be added and medium which contains cells together with one or more biosynthetic products secreted from the cells can be collected on a continuous basis. Mannose-6-phosphate tagged mASA is secreted into the medium and, optionally, purification of rhASA is facilitated by the use of ammonium salts (NH_4Cl) in the fermentation step.

One immediate advantage of this system as compared to a batch system is to allow for an effective production phase extending over longer time. It is therefore within the scope of the present invention to operate the system continuously over a period extending over one week, preferably two weeks, more preferably 3 weeks, even more preferably 4 weeks. The cells can be propagated at 37°C, however it is preferred to reduce the temperature to 33-35°C when the plateau of the production phase is reached in order to increase the productivity of the system.

In one preferred embodiment of the invention, this system is based on the use of bioreactors with a volume of 1 L. Alternatively, bioreactors with a volume of 5L or bioreactors capable of holding approximately 4 L of medium may be preferred. As it can be appreciated, however, the system is also intended as a basis for production of rhASA with a larger capacity and thereby scale-up to large scale production as will be required in the pharmaceutical industry. Other preferred embodiments of the present invention are therefore bioreactors or fermentors with a capacity of 10L, more preferably 50L, even more preferably 200L, and still more preferably 1000L. It is further preferred that the production phase extends for at least two weeks an, additionally, a process wherein 1 to 2 reactor volumes of cell culture are harvested each day is contemplated.

In a preferred embodiment, the process according to the invention is performed using one or more bio-reactors that are equipped with cell retention devices and re-circulation loops.

The cell line used to produce the polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F 10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the cell line selected for expression of the enzyme, and will be apparent to the person of ordinary skill in the art.

In order to control the levels of unwanted proteins in the culture medium it is preferred, however, that the system is developed for culturing the cells in a serum-free medium which only contains recombinant human proteins that have molecular weights less than 10 Kd. The total protein concentration is less then 100 ug/ml. In a preferred embodiment of the invention the cell line is cultured in serum-free Excell 302 medium supplemented with insulin-like growth factor-1 (IGF-1).

A starting material for the purification process may be a crude cell extract but preferably the rhASA is secreted by the cells and is subsequently purified from the cell culture supernatant. The purification process may comprise but is not limited to the following general steps:

1) concentration and diafiltration step,
2) capture step (ion-exchange chromatography),
3) intermediate purification step (chromatography),
4) acidic filtration
5) polishing (chromatography),
6) virus removal,
7) formulation,
8) filling and freeze-drying.

In addition, one or more buffer exchange steps may be incorporated

In a currently preferred embodiment of the invention, the concentration and purification process of the above mentioned step ii) comprises the following steps:

I) concentrating rhASA present in the liquid medium by tangential flow filtration;

II) loading the rhASA containing supernatant obtained in step I on an equilibrated chromatography column and eluting one or more fraction(s) containing rhASA;

III) loading the fraction(s) from step II on another equilibrated chromatography column and eluting one or more fraction(s) containing rhASA;

IV) purifying rhASA present in the fraction(s) from step III by tangential flow filtration;

V) polishing the preparation of rhASA from step IV in one or two or more successive steps, each step comprising loading the preparation on an equilibrated chromatography columns and eluting one or more fraction(s) containing rhASA;

VI) passing the fraction(s) from step V through a viral reduction filter;

VII) formulating the fraction(s) from step VI in order to obtain a preparation of rhASA in a suitable formulation buffer;

VIII) optionally filling the formulated preparation of rhASA into a suitable container and freeze-drying the sample.

It is contemplated that it may not be necessary to perform all the geneal and specific steps of the purification process as outlined above. One could for instance omit the formulation step as well as the filling and freeze-drying if the aim is not to provide a final product suitable for medicinal use.

In step I) of the process the concentration of rhASA in the material is increased with the purpose of obtaining a solution of rhASA in a volume, which is sufficiently small to be conveniently loaded onto the chromatography columns of the subsequent steps of the purification procedure. Preferentially the rhASA is concentrated 5 - 50 times in volume, more preferentially 10 - 20 times by tangential flow filtration. It is obvious to the person of ordinary skill in the art that various different membranes with different nominal weight cut-offs can be used. Nominal weight cut-offs may thus be in the range of 10 to 100 kDa, however, in the present application it is preferred that a 30 KDa membrane is used.

An additional part of step I) includes diafiltration of the rhASA containing solution, which is performed in order obtain a solution of rhASA in a buffer suitable as a loading buffer in the subsequent chromatography steps. Diafiltration is performed using commercially available equipment and following a standard procedure well known to a person skilled in the art.

In a preferred embodiment step II in this process is based on anion exchange chromatography. Anionic resins will generally bind proteins with a net positive charge. Negatively charged or neutral proteins will pass through the matrix, and positively charged proteins (with varying degrees of charge) can be discriminately eluted by gradually changing (in a linear fashion or stepwise linear fashion) the counterion charge of the system with a salt.

As will be known by the person skilled in the art, ion exchangers may be based on various materials with respect to the matrix as well as to the attached charged groups. For example, the following matrices may be used, in which the materials mentioned may be more or less crosslinked: agarose based (such as Sepharose CL-6B®, Sepharose Fast Flow® and Sepharose High Performance®), cellulose based (such as DEAE Sephacel®), dextran based (such as Sephadex®), silica based and synthetic polymer based. For the anion exchange resin, the charged groups which are covalently attached to the matrix may e.g. be diethylaminoethyl (DEAE), quaternary aminoethyl (QAE), and/or quaternary ammonium (Q). In a preferred embodiment of the present process, the anion exchange resin employed is a DEAE Sepharose column, and more specifically, it may be a DEAE Sepharose Fast Flow®, but other anion exchangers can be used.

The ion exchange resin is prepared according to known methods. Usually, an equilibration buffer, which allows the resin to bind its counterions, is passed through the ion exchange resin prior to loading the composition comprising the polypeptide and one or more contaminants onto the resin. Conveniently, the equilibration buffer is the same as the loading buffer, but this is not required.

The aqueous solution comprising the rhASA and contaminant(s) is loaded onto the anionic resin using a loading buffer that has a salt concentration and/or a pH such that the polypeptide and the contaminant bind to the anion exchange resin. The resin is the washed with one or more column volumes of loading buffer followed by one or more column volumes of wash buffer wherein the salt concentration is increased. Finally, the rhASA is eluted by further increasing the salt concentration. Optionally, elution of the enzyme may also be mediated by gradually or stepwise decreasing the pH. The fractions containing rhASA activity are collected and combined for further purification.

It is apparent to the person of ordinary skill in the art that numerous different buffers may be used in the loading, washing, and elution steps. In a preferred embodiment of the invention a loading buffer comprising 5 - 100 mM Tris-HCl, pH 7,0 - 8,0, hereinafter

referred to as "standard buffer", is used. The concentration of NaCl is increased to 0.05 - 0,15 M and 0.2 - 0,4 M during the wash and elution steps, respectively.

In an optional embodiment of the invention, the ion exchange resin is regenerated with a regeneration buffer after elution of the polypeptide, such that the column can be re-used. Generally, the salt concentration and/or pH of the regeneration buffer is/are such that substantially all contaminants and the polypeptide of interest are eluted from the ion exchange resin. Generally, the regeneration buffer has a very high salt concentration for eluting contaminants and polypeptide from the ion exchange resin.

Step III in the purification process comprises an additional chromatography step, which preferably is Hydrophobic Interaction Chromatography (HIC). HIC utilizes the attraction of a given molecule for a polar or non-polar environment and in terms of proteins, this propensity is governed by the hydrophobicity or hydrophilicity of residues on the exposed, outer surface of a protein. Thus, proteins are fractionated based upon their varying degrees of attraction to a hydrophobic matrix, typically an inert support with alkyl linker arms of 2-18 carbons in chain length. The stationary phase consists of small non-polar groups (butyl, octyl or phenyl) attached to a hydrophilic polymer backbone (e.g. cross-linked sepharose, dextran or agarose).

Loading, washing and elution in HIC basically follow the same principle as described above for the ion-exchange chromatography, but often nearly opposite conditions to those used in ion exchange chromatography are applied. Thus, the HIC process involves the use of a high salt loading buffer, which unravels the protein to expose hydrophobic sites. The protein is retained by the hydrophobic ligands on the column, and is exposed to a gradient of buffers containing decreasing salt concentrations. As the salt concentration decreases, the protein returns to its native conformation and eventually elutes from the column. Alternatively proteins may be eluted with PEG.

The use of butyl sepharose and octyl sepharose as solid phases in the HIC is preferred in the present invention. Again, it is readily apparent that, when it comes to the exact conditions as well as the buffers and combinations of buffers used for the loading, washing and elution processes, a large number of different possibilities exist. In a preferred embodiment the column is equilibrated in the standard buffer mentioned above to which has been added 0,25 - 1 M Na_2SO_4. Washing is performed using 1 - 2 column washes of equilibration buffer followed by 1 -5 column volumes of 1,0 - 3,0 M NaCl or 1.8 M Na-acetate in standard buffer pH 7.5. The rhASA is eluted using 0,25 - 0,75 M NaCl or 1 - 5 column washes of 0,9 M Na-acetate in standard buffer pH 7.5.

In a further preferred embodiment of the invention, the purification of rhASA by HIC succeeds the purification by ion-exchange chromatography as performed in the initial capture step. It is contemplated, however, that the two steps could be performed in the reverse order, but this will presumably lead to a lower yield.

A process according to any of claims 14 to 19, wherein purification of the sample in step IV of the purification process is accomplished by tangential flow filtration.

In step IV of the purification process, the rhASA is purified by separation from contaminants according to their size in an acidic environment by tangential flow filtration. The rhASA forms an octamer at low pH with a theoretical molecular weight of 480 kDa and will therefore be retained by a relatively open membrane while most of the contaminants will pass this membrane (Sommerlade et al., (1994) Biochem. J., 297; 123-130; Schmidt et al., (1995) Cell, 82 271-278; Lukatela et al., (1998) Biochemistry, 37, 3654-3664). As the starting material for this process is a suspension of rhASA as eluted from the chromatography column in the previous step of the process the pH in this suspension is adjusted to 4 - 5 by addition of 0.2 - 1 M Na-acetate pH 4.5. Diafiltration is then performed against 1- 10 buffer volumes of Na-acetate pH 4.0 - 5.5 in a manner well known to somebody skilled in the art. The filtration can be performed with the application of several different filter types with nominal weight cut-off values ranging from 20 - 450 kDa, however it is preferred to use a filter with a cut-off value ranging from 100 - 300 kDa. For further processing of the rhASA containing solution the pH is adjusted to a value within the range between 7 and 8 by addition of Tris-base to a final concentration of approximately 20 - 50 mM.

As an alternative to the acidic tangential flow filtration as described above, separation of rhASA from the contaminants can be obtained with acidic gel filtration using essentially the same conditions and compositions of buffers. The filtration is performed at low pH through a gel filtration column, which has been equilibrated with a solution at low pH. In the current procedure a 0.2 - 0,9 M solution of Na-acetate at pH 4 - 5. As an option, the solution of rhASA is concentrated by tangential flow filtration through a 20 - 50 kDa filter prior to the gel filtration. The extent of concentration may vary considerably so that the rhASA may be concentrated from about 0.1 mg/ml to about 50 mg/ml preferably to about 5 mg/ml.

In a currently preferred procedure, the sample pool from step III is concentrated against a Biomax A-screen, 30 kDa. Diafiltration is performed agianst 3 - 5 column washes of 20 mM Na-acetate, pH 5.4 - 5.7. Most preferred is a process wherein tangential flow filtration occurs against a Biomax A-screen.

Several options exist for the polishing step (step V in the purification process). This step may involves purification using ion-exchange chromatography essentially as described above. As of convenience the sample is loaded in the buffer from the previous step of the purification process. In another embodiment, chromatography on a ceramic hydroxyapatite column can be used.

Hydroxyapatite (HAP) usually refers to the crystalline form of calcium phosphate. The mechanism of HAP involves non-specific interactions between negatively charged protein carboxyl groups and positively charged calcium ions on the resin, and positively charged protein amino groups and negatively charged phosphate ions on the resin. Basic or acidic proteins can be adsorbed selectively onto the column by adjusting the buffer's pH; elution can be achieved by varying the buffer's salt concentration. Again, it is evident that numerous buffer compositions as well as combinations of buffers can be employed. Preferably, however, the column is equilibrated with 1 -10 column washes of a buffer comprising 1 - 100 mM Tris-HCl pH 7,0 - 8,0. As of convenience the sample is loaded in the buffer from the previous step of the purification process. The column is washed with 1 -10 column volumes of the buffer used for equilibration and the sample is eluted in a mixture of this buffer and a buffer comprising 100 - 800 mM Sodium phosphate. Optionally the column is reconstituted by washing with 1 -10 column volumes of 100 - 800 mM Sodium phosphate.

In the chromatography steps the appropriate volume of resin used when packed into an chromatography column is reflected by the dimensions of the column, i.e. the diameter of the column and the height of the resin, and varies depending on e.g. the amount of protein in the applied solution and the binding capacity of the resin used. However, it is within the scope of the present invention to increase the scale of the production process as well as the purification process in order to obtain production and purification of rhASA on an industrial scale. Accordingly parameters such as column size, diameter, and flow rate can be increased in order to comply with the speed and efficiency of such large-scale production. Whereas columns with a diameter ranging from 50 - 100 mm, volumes in the size of 100 - 300 ml, and flow rates between 40 - 400 cm/hour or 5 to 100 ml.

In a presently preferred version, the procedure of step V is based on the characteristics of rhASA as follows: Theoretically and practically, rhASA has a isoelectrical point which is in the vicinity of pH 6.0. This means that the protein should bind to an anion exchanger at apH larger than 6.5 and to a kation exchanger at a pH less than 5.5. The present inventors have confirmed such binding to a kation exchanger experimentally, where it is found that, in principle, no rhASA binds at a pH of 5.6. Approximately 50% of any rhASA binds at at

pH 5.2 and 100% binds at pH 4.8. For the anion exchanger, however, rhASA binds at any pH-value tested within the range from 7.2 down to 4.8. In principle, it thusappears that rhASA binds to positively charged anionexchangers at pH 4.8, where the enzyme itself should also be positively charged. Under the same conditions the enzyme binds equally well to a cationexchanger. It is assumed that these unexpected binding characteristics results from the enzyme being extremely polarized having an strong "positively charged" side and a strong "negatively charged" side. Alternatively, the binding characteristics may be explained by the enzyme changing from a dimer to a octamer at pH-values under 5.8.

The currently preferred procedure of step 5 takes advantage of these unexpected characteristics. The polishing step is thus initiated at pH 6.0 where the enzyme will not bind to a first cation exchanger (passive step).This step, however, eliminates many of the contaminating Host Cell Proteins. For a preparation to be used in a pharmaceutical preparation the amount of such proteins must be at a very minimum.

Following the passage through the cation resin the rhASA will bind to a subsequent anion exchange resin which may be coupled to the cation resin in series. If the two resins are coupled, the cation exchange resin is subsequently uncoupled and the anino exchanger is washed at a pH around 4.8, leaving rhASA bound to the resin. The fact that rhASA remains bound to the resin at pH 4.8 is highly unexpected and it is contemplated that this ability will only be shared by very few other protein as, effectively they should have an isoelectrical point below 4.2.

Accordingly, in the currently most preferred embodiment of the invention the two or more successive steps in step V of the purification process comprise a passive step, wherein the rhASA passes through an affinity chromatography resin or membrane, and an active step, wherein the rhASA is detained within and subsequently eluted from an anion exchange membrane or resin. The anion exchange membrane or resin may be a high resolving anion exchanger.
Additionally, it may be further preferred that the affinity chromatography chromatography membrane or resin and said anion exchange membrane or resin are coupled in a series.

In acurrently mos preferreed embodiment of the invention the affinity chromatography chromatography membrane or resin is a Mustang™ S membrane or a Blue Sepharose resin and said anion exchange membrane or resin is a Mustang™Q membrane or Resource™ Q resin.

In a specific embodiment of the invention, the columns are equilibrated with more than 10 column volumes of 20 - 100 mM NA-acetate pH 4.5-6.0. Sample pool from step 4 is loaded

on the columns and after passage of the rhASA through the cation exchange column the two columns are uncoupled and the anion exchanger is washed with 2-4 column volumes of 50 - 75 mM Na-acetate pH 4.8. The anion exchanger is equilibrated with more than 10 column volumes of 20 mM Tris-HCL pH 7.5 (standard buffer). The column is washed with 0.1 mM NaCl in standard buffer and the rhASA is eluted with a linear gradient of 0.1 - 0.3 M NaCl in standard buffer.

In the purification process is further incorporated one or more steps of virus inactivation or virus filtration. It is understood that these methods are intended to give rise to a preparation of an enzyme, which is substantially free of infectious vira and which can be denoted a "virus-safe product". In addition, it is contemplated that the various methods can be used independently or in combination. Preferably, virus filtration is performed after purification of the enzyme by several steps of chromatography. In a preferred embodiment, the virus filtration step is performed by passage of the rhASA containing solution which is a result of step 5 of the purification process through a sterile filter and subsequently passage of the sterile filtered solution through a nanofilter. By "sterile filter" is meant a filter, which will substantially remove all micro-organisms capable of propagating and/ or causing infection. Whereas it is preferred that the filter has a pore size of 0.1 micron, the pore size could range between 0.05 and 0.3 micron.

In addition to or as an alternative to virus filtration, virus-inactivation can be accomplished by the addition of one or more "virus-inactivating agents" to a solution comprising the enzyme. Preferably, the virus-inactivating step will be performed prior to the purification process in order to assure that the agent is not present in the final product in any amounts or concentrations that will compromise the safety of the product when used as a pharmaceutical or when the product is used for the preparation of a pharmaceutical. The term "virus-inactivating agent" is intended to denote such an agent or a method, which can be used in order to inactivate lipid-enveloped viruses as well as non-lipid enveloped viruses. The term "virus-inactivating agent" is to be understood as encompassing both a combination of such agents and/or methods, whenever that is appropriate, as well as only one type of such agent or method.

Preferred virus-inactivating agents are detergents and/or solvents, most preferably detergent-solvent mixtures. It is to be understood that the virus inactivating agent is optionally a mixture of one or more detergents with one or more solvents. A wide variety of detergents and solvents can be used for virus inactivation. The detergent may be selected from the group consisting of non-ionic and ionic detergents and is selected to be substantially non-denaturating. Preferably, a non-ionic detergent is used as it facilitates the subsequent elimination of the detergent from the rhASA preparation in the subsequent

purification steps. Suitable detergents are described, e.g. by Shanbrom et al., in US Patent 4,314,997, and US Patent 4,315,919. Preferred detergents are those sold under the trademarks Triton X-100 and Tween 20 or Tween 80. Preferred solvents for use in virus-inactivating agents are di- or trialkylphosphates as described e.g. by Neurath and Horowitz in US Patent 4,764,369. A preferred solvent is tri(n-butyl)phosphate (TNBP). An especially preferred virus-inactivating agent for the practice of the present invention is Tween 20, but, alternatively, other agents or combinations of agents can be used. The preferred agent added in such a volume that the concentration of Tween-20 in the rhASA-containing solution is within the range of 0.5 - 4.0% by weight, preferably at a concentration of about 1% by weight.

The virus-inactivation step is conducted under conditions inactivating enveloped viruses resulting in a substantially virus-safe rhASA-containing solution. In general, such conditions include a temperature of 4-37°C, such as 19-28°C, 23-27°C, preferably about 25°C, and an incubation time found to be effective by validation studies. Generally, an incubation time of 1-24 hours is sufficient, preferably 10-18 hours, such as about 14 hours, to ensure sufficient virus inactivation. However, the appropriate conditions (temperature and incubation times) depend on the virus-inactivating agent employed, pH, and the protein concentration and lipid content of the solution.

It is contemplated that other methods for removal of or inactivating virus can also be employed to produce a virus-safe product, such as the addition of methylene blue with subsequent inactivation by radiation with ultraviolet light.

In a preferred embodiment, the process according to the invention results in a product comprising a relative amount of bioactive rhASA which is at least 90%, such as at least 95%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 99,9% of the total amount of proteins as determined by reverse phase HPLC.

A therapeutic formulation comprising the polypeptide, optionally conjugated with a heterologous molecule, may be prepared by mixing the polypeptide having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. "Pharmaceutically acceptable" carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyidimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride : phenol, butyl or benzyl alcohol; alkyl parabens such as

methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecularweight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e. g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN, PLURONICST or polyethylene glycol (PEG).

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly- (methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particlesand nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences l6th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide variant, which matrices are in the form of shaped articles, e. g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly (2-hydroxyethyl-methacrylate), or poly (vinylalcohol)), polylactides (U. S. Pat. No. 3,773,919), copolymers of L-glutamic acid and y ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT TM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D- (-)3-hydroxybutyric acid.

The polypeptide purified as disclosed herein or the composition comprising the polypeptide and a pharmaceutically acceptable carrier is then used for various diagnostic, therapeutic or other uses known for such polypeptides and compositions. For example, the polypeptide may be used to treat a disorder in a mammal by administering a therapeutically effective amount of the polypeptide to the mammal.

In a specific embodiment of the invention, the rhASA is formulated in an isotonic solution such as 0.9% NaCl and 10-50 mM Sodium phosphate pH 6.5 - 8.0 or sodium phosphate, glycine, mannitol or the corresponding potassium salts. In another embodiment the rhASA is formulated in a physiological buffer, such as:

a) formulation buffer I containing (in mM): Na_2HPO_4 (3.50 - 3.90), NaH_2PO_4 (0 - 0.5), Glycine (25 - 30), Mannitol (230 - 270), and water for injection; or
b) formulation buffer II containing (in mM): Tris-HCl (10), Glycine (25 - 30), Mannitol (230 - 270), and water for injection.

In a further embodiment, the rhASA is formulated as lipid vesicles comprising galactoside and/or phosphatidyl choline and/or phosphatidyl ethanolamine.

A further embodiment of the invention is a process wherein the rhASA is formulated as a sustained release formulation involving bio-degradable microspheres, such as microspheres comprising polylactic acid, polyglycolic acid or mixtures of these.

It may further be desired to formulate the rhASA with a hypertonic solution in order to cause osmotic opening of the blood-brain barrier and also to formulate the rhASA is formulated in a solution comprising an enhancer for nasal administration.

A specific embodiment is contemplated, wherein the rhASA is formulated so as to enhance its half-life in the bloodstream and/or reduce clearing via the kidneys and/or prevent extended uptake via the liver.

Finally, the process according to the invention may result in production, purification and formulation of a protein, which is enzymatically equivalent to rhASA. This enzyme may in terms of its structure be different from the rhASA according to SEQ ID NO: 2.

As the active form of rhASA within the lysosymes is an octamer a further object of the invention is to provide a rhASA which is an octamer or assembles into an octamer under physiological conditions.

Other aspects of the present inventions include analytical methods for testing the efficiency of each purification step as well as the quality of the resulting preparations of rhASA with respect to, for instance, enzyme activity, concentration of total protein and rhASA, purity, and endotoxin levels.

Enzyme activity, which is to be understood as the catalytic activity of the rhASA, may be measured in an enzyme assay based on the rhASA mediated hydrolysis of either a detectable substrate or a substrate, which leads to a detectable end product. In a preferred aspect the assay is based on hydrolysis of the synthetic, chromogenic substrate, para-Nitrocatechol sulphate (pNCS) which has an end product, para-Nitrocatechol (pNC) that absorbs light at 515 nm.

Total protein concentration in in-process samples and final products may be determined by a commercially available assay that utilises principles of the reduction of Cu^{2+} to Cu^{+} by proteins in an alkaline medium (the Biuret reaction). This method is well known to a person skilled in the art.

Concentration of rhASA in samples collected after various steps of the purification process may be assessed in rhASA enzyme linked immunosorbent assay (ELISA). Quantitative determination of a protein by ELISA is a conventional technique known to the person of ordinary skill in the art. However, it is within the scope of the present invention to provide a specific ELISA for the detection of rhASA based on capturing the enzyme with specific polyclonal immunoglobulins and subsequently detecting the captured enzyme with specific monoclonal antibodies.

Purity and identity of the various preparations of rhASA may be determined by methods well known to the person of ordinary skill in the art, such as rpHPLC, SDS-PAGE, and Western blot rhASA. In addition, the amount of whole cell proteins (HCP) in preparations of rhASA may be determined by the use of ELISA as well as Western blotting techniques using commercially available antibodies.

Preferably, all the above mentioned processes are adapted to be performed in microtiter plates for conveniency.

The product of the present invention has as one of its characteristics a very low content of host cell proteins. In a product intended as a pharmaceutical composition or a product intended to be used in the preparation of a pharmaceutical composition the content of such proteins is critical since they are expected to have immunogenic effects. In a preferred embodiment of the invention the final product contains less than 1,5% whole cell proteins, such as less than 1%, e.g. less than 0.75%, or less than 0,5%, or less than 0,25% whole cell proteins. The product may further contain impurities in the form of enzymatically inactive variants of the main component. In a preferred embodiment the product contains at least 90% enzymatically active rhASA, such as 92% or 94%. In an even more preferred embodiment the relative amount of enzymatically active rhASA is at least 90%, such as 95% or 97% or even 98% or 99%.

In addition to the process according to the invention it is an object of the invention to provide a pharmaceutical composition comprising rhASA, which is efficiently endocytosed via the mannose-6-phosphate receptor pathway in vivo. When applied to endocytosis of rhASA, the term "efficiently" refers to endocytosis leading to at least a 100-fold increase in

the concentration of rhASA within cells in the kidney, the plexus brachialis and the nervus ischiaticus in MLD mice eight days after intravenous injection of a single dose of rhASA (40 mg/kg).

In accordance with the described efficiency of the of the purification process it is preferred that this composition comprises at least 98% bioactive rhASA as determined by reverse phase HPLC. A further characteristic of the enzyme prefared according to the process of the invention is its high specific activity. It is thus preferred that the pharmaceutical composition comprises a rhASA with a specific activity of at least 10 u/mg, at least 20 u/mg, at least 25 u/mg, at least 30 u/mg, at least 40 u/mg, at least 50 u/mg, at least 75 u/mg, at least 100 u/mg, at least 150 u/mg, at least 200 u/mg at least 250 u/mg or at least 300 u/mg.

The pharmaceutical composition according to the invention may comprise a peptide or polypeptide capable of facilitating the entry of rhASA into the central nervous system. It is an object of the present invention, however, to provide an rhASA or a pharmaceutical composition comprising an rhASA which is capable of correcting the abnormalities in sulfatide levels without being associated with such peptides or polypeptides. Accordingly, it is preferred that the pharmaceutical composition does not comprise a peptide or polypeptide capable of facilitating the entry of rhASA into the central nervous system.

A putative system for delivery of rhASA includes the delivery of cells which containing exogenous rhASA. Thus the pharmaceutical composition according to teh invention may comprise intact cells.

In accordance with the above preference, however delivery of the rhASA according to the invention shall preferably not rely on the use of other types of delivery vehicles and, accordingly, it is preferred that the pharmaceutical composition does not comprise intact cells.

Its may be preferred that the rhASA is a rhASA, which is obtainable by the process according to any of claims 1-35.

Another main aspect of the invention is rhASA as a medicament, wherein the rhASA may have any of the characteristics described above. Specifically, it is an object of the invention to provide a rhASA as a medicament for reducing the galactosyl sulphatide levels within target cells in the peripheral nervous system and/or within the central nervous system in a subject.

In accordance with the disclosures in the present application the administration of this rhASA will lead to decreased impairment of motor-learning skills and or to increased nerve motor conduction velocity and/or nerve conduction amplitude.

Yet another main aspect of the invention is the use of a rhASA for the manufacture of a medicament for reducing the galactosyl sulphatide levels within target cells in the peripheral nervous system and/or within the central nervous system in a subject. In accordance, with the above description this rhASA may be obtainable by a process according to the invention.

Still another aspect of the invention is a method of treating a subject in need thereof, said method comprising administering to said subject a pharmaceutical composition comprising a rhASA, which may have any of the characteristics described above, and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within said subject.

In one preferred embodiment of the invention the method comprises administering said pharmaceutical composition intravenously to said subject and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within the peripheral nervous system in said subject.

According to a recent publication intrathecal injection, that is injection directly into the cerebrospinal fluid) of recombinant human alpha-L-iduronidase (rhIDU) can reduce corbohydrate storage in brain tissue in a canine model of mucopolysaccharidosis (MPL) (Kakkis, 2003). On the basis of these observations, the present invention provides methods of treatment which comprise:

(a) administration of a pharmaceutical composition according to the invention intravenously in order to obtain a reduction in galactosyl sulphatide levels in target cells within the peripheral nervous system, optionally as an adjunct to treatment by bonemarrow transplantation; and/or

(b) administration of a pharmaceutical composition according to the invention intravenously in order to obtain a reduction in galactosyl sulphatide levels in target cells both within the peripheral and central nervous system; and/or

(c) administration of a pharmaceutical composition according to the invention intravenously in order to obtain a reduction in galactosyl sulphatide levels in target cells within the peripheral nervous system, optionally in combination with intrathecal injection of a pharmaceutical composition according to the invention in order to obtain a reduction in galactosyl sulphatide levels in target cells within the central nervous system.

Accordingly, the method according to the invention preferably comprises administering said pharmaceutical composition intravenously and by spinal injection and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within the peripheral nervous system and in target cells within the central nervous system in said subject.

According to a further preferred embodiment of the invention the method comprises administering said pharmaceutical composition intravenously to said subject and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within the peripheral nervous system and in target cells within the central nervous system in said subject.

A further preferred embodiment of the invention is a method wherein rhASA is efficiently endocytosed in vivo into target cells within a tissue selected from the group comprising liver, kidney, spleen, heart.

As the skilled addressee will be aware said target cells within the central nervous system are preferably oligodendroglia and said target cells within the peripheral nervous system are preferably Schwann cells.

It is contemplated that the exact nature of the treatment plans based on the method according to the present invention will depend on factors such as age, sex and disease stage of the subject to be treated, and that the optimal dosis regimen and frequency of administration may, with advantage, be determined on an empirical basis. However In one preferred embodiment of the inveniton said pharmaceutical composition is administered in one or more doses, each dose comprising an amount of rhASA which is within the range of 0,1 to 100 mg/kg body weight, such as within the range of 0,25 to 50, 1 to 25, 1 to 10 or 1 to 5 mg/kg body weight.

Also it may be preferred that the pharmaceutical composition is administered on a daily, weekly, bi-weekly or monthly basis.

In accordance with the above description, intravenous and or spinal injection of said pharmaceutical composition may preferably be performed as a supplement to bone marrow transplantation.

While it is normally believed that the active components of therapeutical compositions must be able to be delivered directly into the central nervous system or alternatively must diffuse or be transported across the blood brain barrier in order to have an effect occurring within the central nervous system in order. In a further preferred embodiment of the

present invention, however the method according to the invention leads to a reduction in the galactosyl sulphatide levels in target cells within the central nervous system is created wholly or in part to a washout effect caused by the clearance of sulfatides in tissues and in blood. Sulfatides are cleared from the brain due to the concentration gradient resulting from the clearance of sulfatides in the tissue and in the blood.

In a further embodiment of the invention, the pharmaceutical composition further comprises a hypertonic solution or is administered together with a hypertonic solution in order to cause osmotic opening of the blood-brain barrier.

The ability of rhASA to enter into a target cell over the cell membrane while maintaining its catalytic properties can be determined in vitro using cultures of a cells containing a suitable substrate for the enzyme as described by Kudoh and Wenger. J. Clin. Invest. 1982. 70(1): 89-97. Example 2 of the present text provides an example of such a model system and shows test results for a preparation of rhASA obtained by the process described.

Accordingly, one aspect of the present invention includes a model system comprising a culture of mammalian cells wherein the degradation of sulfatides by exogenous ASA can be determined. In a preferred aspect of this embodiment these cells are fibroblasts derived from a MLD patient and accordingly they lack endogenous functional ASA. It is readily apparent, however, that other cells which are characterised by deficient ASA activity and the ability to accumulate a detectable substrate of ASA can form the basis of such an assay, hereunder cells that are genetically engineered so as to reduce or abolish the expression and/or function of ASA. In another preferred aspect of this embodiment the sulfatides used as substrate in this system are labelled by the addition of a radioactive or non-radioactive compound for subsequent detection. In a most preferred aspect the substrate of rhASA is palmitoyl sulfatide, and in an equally preferred aspect the substrate is labelled with ^{14}C.

An additional embodiment of the invention is a preparation of rhASA, which is capable of reducing the cellular content of one or more of its substrates when analysed in the system described above. Preferably, the enzyme will be added to the culture medium at a concentration ranging from 0 to 100 mU/ml and lead to a reduction in the intracellular substrate levels preferably corresponding to 20%, more preferably 30%, even more preferably 40%, still more preferably 50%, yet more preferably 60%, most preferably more than 70%.

A final aspect of the invention provides a continuous cell culture system as described for the process according to the invention.

With respect to the above description of the various aspects of the present invention and of the specific embodiments of these aspects it should be understood that any feature and characteristic described or mentioned above in connection with one aspect and/or one embodiment of an aspect of the invention also apply by analogy to any or all other aspects and/or embodiments of the invention described.

When an object according to the present invention or one of its features or characteristics is referred to in singular this also refers to the object or its features or characteristics in plural. As an example, when referring to "a cell" it is to be understood as referring to one or more cells.

Throughout the present specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The following text provides an example of production of rhASA by continuous cell propagation in the culture system and purification of the product according to step 1 through 5 of the purification procedure as outlined above (examples 1 and 2). The biological activity of the purified rhASA is evaluated in an in vitro system as described above. Furthermore, the effects of administrating rhASA according to the present invention in vivo are evaluated in a series of experiments performed on ASA(-/-) (knockout) mice, also denoted MLD-mice. Results from these *in vitro* and *in vivo* experiments are provided in examples 3 to 6.

These examples serve to provide a further characterisation of the invention, however they are not intended to be limiting to the scope of the invention.

BRIEF DESCRIPITON OF THE ACCOMPANYING DRAWINGS

Figure 1: Schematic representation of the system for continuous cell propagation.

Figure 2: Ion exchange chromatography batch test of rhASA

Figure 3: (A) HPLC-chromatogram of rhASA after completion of step (V) of the purification procedure according to the present invention. (B) Enlargement of the HPLC chromatogram of (A).

Figure 4: Sulfatide clearance in MLD-fibroblasts loaded with radiolabelled sulphatide after incubation with rhASA for 24 hours.

Figure 5: Sulfatide clearance in MLD-fibroblasts loaded with radiolabelled sulphatide after incubation with rhASA for 6, 24 and 48 hours.

Figure 6: rhASA serum levels 10 min after intravenous injection of rhASA

Figure 7: Plasma kineticts in MLD mice following one intravenous injection of 20 mg/kg rhASA.

Figure 8: Sulfatide levels in kidney following one iv rhASA administration to MLD mice.

Figure 9: rhASA concentrations in (**A**) kidney, (**B**) plexus brachialis and (**C**) nervus ischiaticus of ASA knock out mice (ASA-/-) 8 days after a single intravenous injection of 10, 20 and 40 mg/kg rhASA. Untreated wildytpe mice (ASA+/+) and knock out mice injected with buffer alone (0 mg/kg) were used as controls and show no significant immunoreactivity for the human enzyme (in C low signals in the control groups are due to an increased background caused by prolongated incubation times with antibodies to increase the sensitivity of the assay); the means +/- SDs are shown (n=5)

Figure 10: Lipid levels in (**A**) kidney, (**B**) plexus brachialis and (**C**) nervus ischiaticus in treated ASA knock out mice and controls as indicated; the levels of sulfatide (open bars), sphingomyelin (black bars) and cholesterol (grey bars) were determined by densitometric scanning of stained TLC plates; means +/- SDs, n=5; sulfatide levels which differ significantly from those of mock-treated ASA knock out mice are indicated by asterisks (one-way ANOVA; $P < 0.05$)

Figure 11: Sulfatide levels in kidney following one to four weeks intravenous rhASA administration to MLD mice receiving one weekly dose of 20 mg/kg. The levels of sulfatide (open bars), sphingomyelin (black bars) and cholesterol (grey bars) were determined by densitometric scanning of stained TLC plates; means +/- SDs, n=3;

Figure 12: Sulfatide levels in plexus brachialis following one weekly dose of 20 mg/kg for one to four weeks intravenous rhASA administration to MLD mice. The levels of sulfatide (open bars), sphingomyelin (black bars) and cholesterol (grey bars) were determined by densitometric scanning of stained TLC plates; means +/- SDs, n=3;

Figure 13: Sulfatide levels in nervus ischiaticus following one weekly dose of 20 mg/kg for one to four weeks intravenous rhASA administration to MLD mice. The levels of sulfatide (open bars), sphingomyelin (black bars) and cholesterol (grey bars) were determined by densitometric scanning of stained TLC plates; means +/- SDs, n=3;

Figure 14: Sulfatide levels in brain following one weekly dose for four weeks intravenous rhASA administration to MLD mice. the levels of sulfatide (open bars), sphingomyelin (black bars) and cholesterol (grey bars) were determined by densitometric scanning of stained TLC plates; means +/- SDs, n=3;

Figure 15 : Data from rotarod studies on rhASA and placebo treated MLD mice and wild type control mice (**A**) before treatment, and (**B**) one month after administration of the first dose in the treatment plan.

Figure 16: Presentation of electrophysological parameters from studies on nerve motor conductivity.

EXAMPLES

Example 1: Continuous cell propagation

A continuous cell propagation system and a small to medium size purification process for rhASA in 200 - 400 ml column scale intended for scale-up to large-scale production (column scale >2L) is developed. A schematic representation of the system is given in figure 1. The quality and purity of the final product (rhASA) is very high and suitable for toxicology testing (including steps 1-4 + 7) and finally also suitable for clinical trials (including all steps described). As described above, the process will include a capture step, 1-2 intermediate purification steps, 1 polishing step, 1-2 virus removal steps and 1 formulation step. 1 or more buffer exchange steps will also be included.

Experimental design: Several different chromatography gels are tested and performance of the different steps (with respect to removal of contaminants, yield and purity) are analysed with a battery of analytical methods described briefly below.

Analytical methods

Enzyme activity:	Arylsulfatase assay
Total protein concentration:	BCA analysis

rhASA concentration:	rhASA ELISA
Purity:	rpHPLC, SDS-PAGE
Identity:	rpHPLC, Western Blot rhASA
HCP proteins:	HCP-ELISA, Western blot HCP proteins
Endotoxin level:	According to European Pharmacopoeia (Ph. Eur.) method 2.6.14. For i.v.-administration the acceptable value is 5 IU/kg/h. With a maximal dose of 1 mg/kg/h and a concentration of the product of 5 mg/ml, the limit is 25 IU/ml.
Osmolality:	According to Ph. Eur. method 2.2.35. Since no acceptable value is stated in the European Pharmacopoeia for this exact product the value (250-350 mOsmol/kg) is defined because it compares to an isotonic solution of (0.9%) NaCl, which is well-tolerated in-vivo.
DNA content:	DNA threshold
pH:	According to Ph. Eur. method 2.2.3. Since no acceptable value is stated in the European Pharmacopoeia for this exact product. The value (7.0 – 8.0) is defined because it is neutral pH and well-tolerated in-vivo.
Bacterial count:	Ph. Eur. method 2.6.12 (membrane filtration) will be used to test the API and Bulk Substance. There is no acceptable value stated in the European Pharmacopoeia for this exact product. The value (≤ 10 cfu/ml) is defined to ensure an adequate minimal bioburden prior to sterilisation. The final product for i.v.-administration will be sterile and tested according to Ph.Eur. method 2.6.1

Description of analytical methods

Arylsulphatase Assay

In addition to its natural substrates ASA is also able to catalyze the hydrolysis of the synthetic, chromogenic substrate, para-Nitrocatechol sulfate (pNCS), see Fig. . The product, para-Nitrocatechol (pNC), absorbs light at 515 nm. The method is described by Fluharty et al. 1978, Meth. Enzymol. 50:537-47

Materials and Equipment

Spectrophotometer Spectra MAX Plus from Molecular Devices or equivalent.
Cuvette 1 ml (glass or plastic) with 1 cm path-length suitable for 515 nm.
Flat bottomed 96 well micro-titer plate.

Chemicals and Reagents

pNCS - p-NitroCatechol Sulfate (no.N-7251, Sigma)
BSA - Bovine Serum Albumin Frac. V
NaAc – Sodium Acetate trihydrate
Triton X-100
Tris-HCl molecular biology grade
PBS, pH 7.4 w/o Ca^{2+}, Mg^{2+}: 0.20 g/l KCl, 0.20 g/l KH_2PO_4, 8 g/l NaCl, 1.15 g/l Na_2HPO_4. Adjust pH.

All other solvents and chemicals were of p.a. quality (Merck)

a. 2X ASA substrate solution: 30 mM pNCS, 10 % (w/v) NaCl and 1 mg/ml BSA in 0.5 M NaAc pH 5.0.
b. TBS, pH 7.5: 10 mM Tris-HCl and 150 mM NaCl in H_2O.
c. Stop solution: 1 M NaOH

Since many anions and kations, such as SO_4^{2-}, PO_4^{3-}, SO_3^{2-}, F^-, Ag^+, $Cu^{2+}$and Hg^{2+}, are inhibitors of the enzyme at concentrations in the millimolar range or lower the sample is transferred to a suitable buffer (e.g. TBS) before activity is measured. This is done by dialysis or buffer exchange on a gel filtration column (e.g. PD10 from Amersham Pharmacia Biotech).

a. Measurement of ASA activity in cell supernatants

The used medium is centrifuged (110 x g, 5 minutes) and the supernatant is transferred to a clean tube. The buffer is changed to TBS by dialysis or by using a gel filtration column.

b. Measurement of intracellular ASA activity

Cells in suspension are washed once with PBS and then once with TBS before they are lysed in 0.5 ml TBS+0.5 % TritonX-100 for 10 minutes, RT. After vortexing the lysates are centrifuged (13.200 rpm, 10 minutes) and supernatants collected in clean tubes. Alternatively, the cells are resuspended in TBS and then lysed by repeated freeze-thawing cycles.

c. Measurement of ASA activity in in-process samples and final product

The buffer is changed to TBS before activity is measured and protein concentration in the samples is determined using the BCA Protein Assay Reagent kit (see below). In order to assure linearity a final absorbance between 0.1 and 2 (see reference 2) is aimed at. Samples are diluted in TBS if necessary.

a. 50 µl of sample diluent (TBS or TBS+TritonX-100) is added in at least duplicates to a micro-titer plate and use as blanks.

b. 50 µl of samples or diluted samples is added in duplicates to the micro-titer plate.

c. 50 µl of 2X ASA substrate solution is added into each well. The plate is sealed and incubated at 37° +/- 0.5°C for exactly 30 minutes.

d. The reaction is stopped by adding 50 µl of stop solution (1 M NaOH) into all wells.

e. Pre-read is done using a micro-titer plate filled with 0.15 ml MilliQ water/well to correct for scattering effects. Subsequently the absorbance at 515 nm is measured within 30 minutes using a plate reader. The absorbance measured from the micro-titer plate to a 1 cm path length by the use of an application named Path Check.

f. The delta absorbance (ΔA) is calculated by subtracting the absorbance value of the blank from the measured absorbance of each of the samples. The molar extinction coefficient (εM) for the product pNC is 12 400 M-1 cm-1.

Calculations

Definition: One Unit (1 U) of enzyme activity is defined as the hydrolysis of 1 µmol pNCS per minute at 37° C, pH 5.0.

The following equation is used in order to calculate the enzyme activity in µmol pNCS hydrolysed / min x ml (=Units/ml):

$$\frac{\text{Vtot (ml)}}{\varepsilon_M /1000 \times \text{Vsample (ml)} \times \text{Incubation time (min)}} \times \Delta A = \text{Units/ml} \quad (1)$$

where:

ΔA = absorbance of sample – absorbance of blank

Vtot (ml) = total reaction volume in ml (in this case 0.15 ml)

Vsample (ml) = added sample volume in ml (in this case 0.05 ml)

ε_M = the molar extinction coefficient for the product pNC, which in this case is 12 400 M^{-1} cm^{-1}

Equation 1 could more simplified be written as:
$\Delta A \times (0.15 / (12\,400/1000 \times 0.05 \times 30)) =$
X μmol / (minute x ml) (=Units/ml) (1)

To calculate the specific activity in μmol pNC consumed/(minute x mg) (=Units/mg) divide equation 1 with the protein concentration of the sample:

Eq. 1 / Protein conc. (mg/ml) = Y μmol / (minute x mg) = Units/mg (2)

BCA analysis

A commercially available assay kit (Pierce BCA Protein assay kit, no. 23225) is used according to the manufacturers instructions.

rhASA ELISA for determination of rhASA concentrations

The procedure is an enzyme-linked immunosorbent assay (ELISA) for quantitative determination of recombinant human Arylsulfatase (rhASA) in solutions, such as buffers, cell culture medium and serum.

rhASA is captured on maxisorp 96-well plates coated with the IgG fraction of rabbit antiserum to affinity-purified rhASA. The captured rhASA is detected with a monoclonal

antibody to rhASA, followed by horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulins. HRP will convert the substrate tetramethylbenzidine (TMB) to a blue product, which turns yellow upon acidification. The absorbance is measured at 450 nm and a standard curve from known rhASA concentrations is used to calculate rhASA concentrations of the samples.

Equipment

Spectrophotometer for plates, i.e. Spectramax Plus, Molecular Devices with SOFTmax PRO software for calculations

Plate washer

Plate shaker

Pipettes; single and multi-channel

Materials

Maxisorp 96-well plates

Sealing tape

Reagents

Coating buffer

Tris-buffered saline (TBS): 10 mM Tris-HCl, 0.15 M NaCl, pH 7.4.

Washing buffer

TBS (coating buffer) is supplemented with 0.1% tween-20.

1 ml tween-20 is added to 1 liter of TBS.

Blocking buffer

SuperBlock blocking buffer in TBS (Pierce).

Dilution buffer

10 ml blocking buffer is added to 90 ml TBS (coating buffer).

Polyclonal immunoglobulins to rhASA

Medium from rhASA-CHO cells is affinity-purified on a column with monoclonal antibody to rhASA (5.7) cross-linked to Protein A. Rabbits are immunized with affinity-purified rhASA (DAKO) and the antisera are verified to react to rhASA with western blotting. Antiserum from rabbit is purified on HiTrap protein G column(s).

The IgG fraction is stored in 50% glycerol, 10 mM Na-Pi, 75 mM NaCl, pH 7.2 at 4^0C. The protein concentration is 1.25 mg/ml, determined with BCA protein assay kit.

rhASA standard

Purified rhASA, batch M0208, is used as a standard. The standard is purified from rhASA-CHO cell supernatant with three consecutive purification steps, DEAE sepharose, HIC octyl sepharose and Mustang Q.

The stock is stored in 50% glycerol, 10 mM Tri-HCl, pH 7.5 at 4^0C. The concentration, determined with BCA protein assay kit, is estimated to 100 µg/ml.

Monoclonal antibody to rhASA

Supernatant from a rhASA monoclonal antibody (mab) producing hybridoma (19-16-3 from Prof. Gieselmann, Bonn) is purified on a HiTrap protein A column.

The mab is stored at -20^0C in 20 mM Na-Pi, 0.145 M NaCl, pH 7.2 (PBS) supplemented with 0.02% sodium azid. A working portion is kept at 4^0C for 6 months.

HRP-anti-mouse immunoglobulins

Horseradish peroxidase-conjugated, affinity-isolated, goat anti-mouse immunoglobulins are purchased from DAKO (P 0447) and stored at 4^0C.

TMB substrate.

The One-Step Substrate system containing 3, 3', 5, 5'-tetramethylbenzidine (TMB) is purchased from DAKO (S 1600) and stored at 4^0C.

Stop solution

1 M H_2S0_4

Method

Coating

The stock of anti-rhASA polyclonal IgG is diluted 1:1000 in TBS to 1.25 µg/ml and 100 µl/well is added to a maxisorp the 96-well plate. The plate is incubated over night at room temperature and washed twice with ~250 µl washing buffer.

Blocking

200 µl of blocking buffer is added per well prior to incubation at room temperature under agitation for at least 15 - 60 minutes.

Capturing of rhASA

100 µl diluting buffer is added to all wells.

rhASA standard: The rhASA standard stock solution is diluted 2000 times in dilution buffer in triplicates 50 ng/ml. Triplicates of 100 µl standard are transferred to the 96-well plate and serial two-fold dilutions are prepared.

Samples: The samples are diluted in triplicates in dilution buffer to estimated rhASA concentration around 25 ng/ml. 100 µl of each sample are transferred to the 96-well plate. 2-8 two-fold dilutions are prepared in the plate.

The plate is incubated for 100 - 140 minutes at room temperature and under agitation and is subsequently washed four times with ~250 µl washing buffer.

Detection with monoclonal antibody

The monoclonal antibody (mab) to rhASA is diluted 1:2000 to 185 ng/ml in dilution buffer and 100 µl is added to each well. The plate is incubated for 70 - 120 minutes at room temperature under agitation and is subsequently washed four times with ~250 µl washing buffer.

Detection of complexed mab with anti-mouse IgG-HRP

Anti-mouse IgG-HRP is diluted 1:2000 to 500 ng/ml in TBS (coating buffer) and 100 µl is added to each well. The plates are incubated for at 70 -120 minutes at room temperature and under agitation and subsequnetly washed four times as above.

Colour development

TMB substrate (100 µl) is added to each well and the plates are incubated for 15 minutes at room temperature without agitation. The reaction by adding 100 µl/well 1 M H_2S0_4 (stop solution) and the absorbance is measured at 450 nm with endpoint reading in the plate spectrophotometer.

Evaluation

rhASA concentrations are calculated using the SOFTmax PRO software (....) according to the manufacturers instructions.

The linear part of the standard curve is plotted using linear regression and the concentration of unknown samples is read from the standard curve.

Reversed phase HPLC for analysis of rhASA

The purity of Arylsulfatase A (rhASA) is determined by reversed phase HPLC, monitoring the UV absorption at 220 nm. The elution is obtained with an increasing concentration of organic modifier (acetonitrile) in the mobile phase. The retention times for rhASA and other components in the sample are dependent on their ability to adsorb and desorb to the

non-polar stationary phase, which in turn depends on factorssuch as protein conformation, hydrophobicity and sequence.

Materials and Equipment

Hewlett Packard model 1090 HPLC system equipped with a tertiary pump system, auto injector, diode array detector, controlled by HP Chemstation version A.06.03. Equivalent HPLC systems may be used provided that the system suitability test verifies an adequate performance.

Filter for sample concentration: Centriplus YM-30, Millipore corp.

Analytical column: Zorbax 300SB-C18, 2.1*150mm 5-micron, Rockland Technologies Scientific, Inc.

Inline filter: Inline filter A-102X and inline filter cartridge 1*1 mm, Upchurch Scientific, Inc.

Filter for sample preparation: Whatman Anatope 10 LC

Chemicals and Reagents

Milli-Q water, HPLC grade water or equivalent

Acetonitrile, far UV, HPLC grade (VWR, LiChrosolve or equivalent)

Trifluoroacetic acid (TFA), ampoules 10 x 1 g (Pierce)

Tris base p.a. quality (Angus or equivalent)

Guanidinium chloride p.a. quality (VWR biochemistry grade or equivalent)

Mobile phase A: Dissolve 1 ampoule TFA (1 g) in 1 litre of Milli-Q water

Mobile phase B: Dissolve I ampoule TFA (1 g) in 1 litre of acetonitrile

Sample diluent: 20 mM Tris-HCl, pH 7.5

Column cleaning solution 1: 50% Isopropanol p.a. quality in water

Column cleaning solution 2: 6 M Guanidinium choloride

rhASA standard (purified on a mabASA/protein A Sepharose column. Reported in experimental summary Exp. No: M-6). If affinity purified rhASA not is available, samples of lower purity from the rhASA purification scheme may be used as a standard.

All other chemicals and reagents were of p.a. quality if not otherwise stated and purchased through common commercial sources.

Method

Instrumental conditions

Mobile phase composition:	A:	Water, 0.1% TFA
	B:	Acetonitrile (AcN), 0.1% TFA
Flow rate		0.2 ml/min
Temperature:		+40° C

Sample injection volume:

Crude extract	20 µl (if concentrated to 0.3 mg/ml)
In-process samples:	20 µl (if concentrated to 0.3 mg/ml)
Affinity purified samples:	5 µl (if 1.0 mg/ml)

Gradient:

Time (min)	%A	%B
1.00	70	30
10.00	40	60
15.00	5	95
20.00	5	95
25.00	70	30
30.00 (post time)	70	30

Column wash (performed every 5th injection):

Injection of 25µl of 50% isopropanol (p.a. grade) as a sample and a run of the gradient stated above in order to clean the column.

Sample and standard preparation

rhASA samples with a protein concentration less than 100 µg/ml are concentrated in a Centriplus centrifugal filter device (model YM-30, Millipore Corp.).

The obtained retentate is adjusted to a protein concentration of 1.0-0.3 mg/ml with 20 mM Tris-HCl pH 7.5 and filtrated through a 0.22 µm filter in order to remove any particles and

precipitated proteins. In case of small sample volumes the filtration can be replaced by centrifugation at 10.000g for 10 minutes.

Chromatography

The samples are loaded and run on the chromatograph while the temperature is kept low (+8°C) if possible.

Integration and calculation of purity

Area under the curve measured at 220 nm for the rhASA peak is calculated and related to total integrated area. Purity is reported as percentage rhASA of total protein. Use the integration parameters in the appendix (designed for Hewlett Packard/ Agilent Chemstation 06.03 software) as base for integration. Since integration of the rhASA main peak is not always optimal with the preset integration parameters, manual integration might be necessary. Different HPLC software might also require different integration parameters, which has to be tested individually for each system.

Evaluation

Identity: The retention of the main peak of the sample should be within ± 0.5 minutes as compared to the rhASA standard.

Purity: The purity of the sample is determined by comparing the integrated area of the main peak compared to total integrated area. Purity is reported as % main peak (rhASA).

Raw data

Raw data files are stored on a server or CD-ROM discs.

Appendix

Integration parameters

Integration parameters are highly instrument and system dependent and have to be evaluated for different systems used. The integration parameters below are optimized for Agilent/ Hewlett Packard ChemStation HPLC software version 06.03.

Event	Value	Time
Slope sensitivity	10.0	initial
Peak width	0.2	initial
Area reject	5.0	initial
Height reject	1.0	initial
Detect shoulders	drop	initial
Integration	OFF	0.000
Integration	ON	5.000

Outline of continuous cell propagation

The continuous mammalian cell propagation has been developed in B. Braun 5L bioreactors equipped with Bio-Sep cell retention devices from AppliSens. The principle of the process presented schematically in Fig. 2. The mammalian cell are cells capable of amplification and production of foreign proteins as a suspension culture in bioreactors or large-scale fermentors.

During the process development the cell line is maintained and propagated in Excell 302 medium (catalog number 81045 from JRH Biosciences). This is a serum-free medium, which is devoid of proteins of animal or human origin. Furthermore, the medium, which does not contain phenol red, has been supplemented with insulin-like growth factor-1 (IGF-1) and with glucose. The glucose concentration is monitored and adjusted to optimal levels during the process.

The recombinant human ASA produced by the continuous culture process in B. Braun 5L bioreactor is presently expressed in CHO DG44 cells. The amplification of the CHO cells after thawing is initiated in T-flasks and the cells are later transferred to spinner flasks. Before splitting and inoculation of the bioreactor culture, the spinnner culture has a cell density of 1.3 10^6 cells/ml with a viability of 96%.

In preparation for the culture process the cells are transferred from the spinner flasks to the bioreactors. Data on cell densities in the bioreactor before and after inoculation can be deducted from table 1 below. Also typical initial values for viability, glucose, agitation, pH, pO_2 and temperature are reported.

When propagated and maintained as described above the cells do not clump, and propagate and produce as suspension cultures.

Part of the propagation, maintenance and production from the CHO DG44 culture is the harvest of 1 – 2 reactor volumes of media per day. To compensate for the harvest, the culture is supplemented with the same amount of fresh medium per day.

The continuous culture process can be maintained over a period of more than 500 hours, and a production phase of 2 weeks or more is preferable. In order to increase the yield it is desirable to lower the temperature from 37°C to 33 - 35°C once the plateau of the production phase is reached. Cell density values at and above the 1.2 x 10^7 cells/ml are obtained and productivities above 3,0 pg/cell/day resulting in > 20 mg rhASA/L is demonstrated in this system. During the process the parameters; glucose, lactate, glutamine, ammonium and osmolarity are measured and controlled.

Parameters	Values
Volume	4000 ml
Agitation	100 - 165 rpm
Temperature	37° (reduce to 33°C - 35°C)
Re-circulation rate	3 to 4 times the perfusion rate
Separation parameters	CRE above 95%
Bleeding	1/10 of bioreactor volume per day
Glucose	2.15 g/l
Lactate	0.13 g/l
Oxygenation	Pure oxygen sparging + PID parameter adjustments
pO_2	30 - 40%
Perfusion rate	Up to 2 vol/day
Cell viability at inoculation	93%
Cell viability in production phase	90%
Cell density at inoculation	3.6×10^5 cell/ml
Cell density	12×10^6 cells/ml
Specific ASA production	1,5 – 3,0 pg/cell/day
Protein output per bioreactor per day	> 170 mg/day
Steady production state	To be defined

Table 1: Main parameters for the cell culture system. Cell Retention Efficiency (CRE) is a measure, reported as a percentage, of the efficacy with which the cell retention device separates the cells from the medium and bring back the cells to the culture vessel. Bleeding is a deliberate harvest of cell containing medium. Proportional Integral Differential (PID) parameter is relevant when controlling the way a process reaches and maintains defined set-points. Steady production-state is a set of process parameters, chosen because the are believed to support an optimal production. The aim is to maintain the process at these parameters for a longer period, the steady production-state, and harvest product during this period.

Outline of purification process

Step 1: Concentration/Diafiltration with Tangential Flow Filtration (TFF)

20 L of medium (ASA activity in the range of 0.3 – 1.5 U/ml) is clarified through a sequence of depth filters from Millipore (Polygard D5 5 μm + Opticap FF and Opticap 0.45 μm) and concentrated 10 – 20 times in volume on a Sartoflow system with a Sartorius frame (Sartorius). A Millipore Biomax 30 kDa screen type A with 0.1 m^2 area is used. After concentration diafiltration is performed against 20 mM Tris-HCl pH 7.5 (referred to as: standard buffer) until the conductivity is approx. 4 mS/cm. The medium was finally filtered through a Opticap 0.45 μm filter

Expected yield is 90 – 100%

Step 2: Capture step – DEAE sepharose FF (Amhersam Biotech)

Sample from step 1 (corresponding to 50.000 U of total activity) is applied on a 800 ml DEAE sepharose packed in a 70 mm diameter column (Pharmacia Index 70/500) equilibrated with standard buffer. Flow rate is 80-120 cm/hr. Protein bound to the DEAE gel is then washed with 2-3 column volumes (CV) of standard buffer followed by 2-3 CV's of 0.1 M NaCl in standard buffer.

rhASA is eluted with 3-4 CV's of 0.3 M NaCl in standard buffer. Fractions containing rhASA activity are pooled and used for further purification. Normal yield is 90 % and purity approximately 30-40%.

Step 3: Intermediate step 1 – Butyl Sepharose FF (Amhersam Biotech)

Sample pool from step 2 is mixed 1:1 with 1.0 M Na_2SO_4 in standard buffer and applied on a 800 ml octyl sepharose FF packed in a 70 mm diameter column (Pharmacia Index 70/500) equilibrated with standard buffer + 0.5 M Na2SO4. Flow rate is 60 - 100 cm/hr. Column is washed with 1 CV of equilibration buffer followed by 1-2 CV's of 1.8 M Na-Avetate in standard buffer pH 7.5. rhASA is eluted with 1.5 – 2.0 CV's of 0.9 M Na-Acetate in standard buffer pH 7.5 and fractions containing activity are pooled and used for further purification. Normal yield is 90 % and purity 70-80 %.

Step 4: TFF step

Sample pool from step 3 is concentrated to approximately 1 mg/ml with TFF against a Biomax A-screen, 30 kDa. Diafiltration is performed against 3-5 volumes of 20 mM Na-Acetate, pH 5.4 – 5.7. Normal yield is 90 – 100 % and purity the same as the previous step.

Step 5: Polishing step

Currently under development and the most likely options at this stage are listed below:

1. Mustang-S membrane or Blue Sepharose (passive step) + anion exchanger or membrane (active step)

Brief description: A Mustang-S membrane or Blue Sepharose is coupled in series with a high resolving anion exchanger (e.g. Resource-Q from Amhersam Biotech or Mustang Q membrane). The columns are equilibrated with > 10 CV's of 20 - 100 mM Sodium Acetate pH 5.4 – 6.0. Sample pool from step 4 is loaded on the columns (rhASA will pass through the Mustang-S membrane / Blue Sepharose and be captured on the high resolving anion exchanger). The Mustang- S membrane / Blue Sepharose is uncoupled and the high resolving anion exchanger is washed with 2-4 CV's of 50 – 75 mM Sodium Acetate pH 4.8.
Equilibrate anion exchanger with >10 CV's of 20 mM Tris-HCl pH 7.5 (standard buffer). Wash column with 0.1 M NaCl in standard buffer and elute rhASA with a linear gradient of 0.1 – 0.3 M NaCl in standard buffer. Collect active rhASA fractions. Yield: 80 – 100 %). Purity > 97 %.

Step 6: Virus filtration step

Virus filtration will be performed on the product pool from step 5 using a 0.1 micron sterile filter followed by a DV 20 nano filter from Pall with an applied constant pressure of 20 – 50 psi. Estimated flow through in process scale is 25 L/hr.
As an alternative, 1% of Tween 20 or 80 could be applied to the supernatant (contact time 30-60 minutes) before the first concentration and diafiltration step (step 1).

Step 7: Diafiltration / Formulation step

Tangential flow filtration (TFF) against a Millipore Biomax 30 kDa screen type A against 5-10 x volumes of formulation buffer will be performed. The most likely formulation buffers are presented below

Formulation buffer 1.

Na_2HPO_4	3.50-3.90 mM
NaH_2PO_4	0-0.5 mM
Glycine	25-30 mM
Mannitol	230-270 mM

Water for injection (WFI)

Formulation buffer 2.

Tris-HCl	10 mM

Glycine 25-30 mM
Mannitol 230-270 mM
Water for injection (WFI)

The pH and osmolality in both Formulation buffers will be balanced to 7.5 ± 0.2 and 300 ± 50 mOsm/kg respectively. Final protein concentration should be according to the specification (>5 mg/ml).

Step 8: Formulation, Filling

Formulation and dosage form

In the development of the dosage form, the stability of rhASA is an important factor to consider. At present, all stability data points towards an aqueous stabile solution. Freeze-dried powder is currently our back-up strategy.

The options at present are the two different formulation buffers described in step 7: Formulation buffer 1 and 2.

Both these formulations are known to stabilize proteins in aqueous solutions as well as in freeze-dried powders. The pH and osmolality in both Formulation buffers will be balanced to 7.5 ± 0.2 and 300 ± 50 mOsm/kg respectively. Final protein concentration should be according to the specification and in the range 5-20 mg/ml.

The filling of rhASA will be performed in a production unit according to EU GMP practice and in a room classified as Class A. During production the filling zone is monitored with particle count and settle plates. The personnel are regularly trained according to EU GMP and monitored after each production with glove prints. The sterility of equipment and materials are secured by validated sterilization procedures.

Results

Data for preparations of rhASA obtained through a purification procedure as outlined above are presented in tables 1 and 2. In brief, the results show that the overall yield of the purification process correspond to 79% of the rhASA present in the starting material. The purity of rhASA in the resulting preparation corresponds to 98.0 % as determined by reverse phase HPLC. Results are shown in figure 3. Specific conditions for the procedure for which data are shown are as follows:

Step 1 – 4: As described above.

Step 5: A 10 ml Mustang-S membrane is coupled in series with a high resolving anion exchanger (Resource-Q from Amhersam Biotech, 6 ml). The columns are equilibrated with > 10 CV´s of 20 mM Sodium Acetate pH 5.5. rhASA from Tox03HC20 is buffer exchanged to the equilibration buffer and loaded on the columns. After passing the Mustang-S membrane, rhASA will be captured on the Resource-Q column. The Mustang- S membrane is uncoupled and the Resource-Q column is washed with 3 CV's of 75 mM Sodium Acetate pH 4.8.

The Resource-Q column is washed with >10 CV's of 20 mM Tris-HCl pH 7.5 (standard buffer) until the correct pH is reached. The column is washed with 0.1 M NaCl in standard buffer and rhASA is eluted with a linear gradient of 0.1 – 0.3 M NaCl in standard buffer. Fractions containing active rhASA are collected.

Table 1: Purification scheme Tox03HC20, which have been used for evaluation of the polishing step. Enzyme activity in the scheme may vary due to changes of the method during development

Step	**Volume (ml)**	**Total Activity (U)**	**Yiel(% activity)**	**Purity (% by rp-HPLC)**
TFF	7990	54358	n.d.	n.d.
Capture: DEAE	2250	61537 (high?)	n.d	n.d.
Intermediate : Butyl	720	42768	n.d	n.d
TFF	655	49125 (based on average activity 75 U/ml)*	90 % (slightly on the high side)	92 %

n.d. = not determined

Table 2: Result from polishing test development in small scale using Tox03HC20 as start material. Test Mustang-S (passive) + Resource-Q (active) as polishing step

Step	**V lume (ml)**	**Total Activity**	**Total protein**	**Specific activity**	**Yield (% activity)**	**Purity (% by**

		(U)	(mg)	(U/mg)		rp-HPLC)
Start: Tox03HC20	3.2	293	11	26.6	100.	91.2 %
Polishing pool	19.0	249	8.7	28.5 (purification factor of 1.07)	85 % (based on activity) 79 % (based on protein)	98.0 %

Example 2: Test of rhASA for binding to cation exchange resin and anion exchange resin

Experimental description:

rhASA (Tox03HC20) 5 mg/ml was mixed 1:10 with buffers at pH 4.8 - 7.2
Kation exchanger (Unosphere-S, BioRad) + Anionexchanger (DEAE FF, Amhersam Biotech) was portioned in test tubes and equilibrated with 20 mM Na-Acetate pH 4.8, 5.2, 5.6 and 6.05 or20 mM Tris-HCl pH 7.2. (approx. 100 ul IEX media / tube). 170 ul rhASA 1:10 in resp. buffer was added to the the IEX media with the same pH + to empty reference tubes. Mix several times and let sit for approx. 30 minutes. Spin down and measure activity in Supernatant.

Conclusion:

rhASA binds as expected to the cation exchanger, but not to the anionexchanger. Even at pH 4.8 rhASA binds strongly and unexpected to the resin. This binding may be explained by strong polarity or alternativley by a change from dimer to octamer below pH 5.8, which induces changes in exposed charged groups. Results are shown in figure 2.

Example 3: Degradation of natural sulfatides in fibroblasts by rhASA

Dose/response experiment:

Experimental design

Fibroblasts from a MLD patient with null-mutation (GM00243, purchased from Coriell Cell Repository, USA) are grown almost to confluency in 25 cm^2 flasks with medium containing heat-inactivated fetal calf serum (FCS). Cells are loaded with the natural substrate, ^{14}C-palmitoyl sulfatide (15 μM). Following incubation for 40 h the medium is changed to rhASA containing medium (0, 25, 50 and 100 mU/ml affinity-purified rhASA, respectively). After 24 h the cells are harvested and lipid extracts are prepared from the cells by a chloroform-methanol extraction. The lipid fractions are analysed by TLC-chromatography by comparing to radioactively labelled references. The TLC plate is exposed to X-ray film and the different lipid fractions from the TLC plate are quantified using liquid scintillation counting. The data is expressed as percent of radioactivity of remaining and metabolised sulfatides.

Results

The data from this experiment (Table 3 and figure 4) shows that all dose levels of rhASA used (0.25, 2.5, 25, 50 and 100 mU/ml) metabolise approximately 40 - 70% of the ^{14}C labelled sulfatide loaded into the MLD fibroblasts. The background degradation of the substrate is approximately 15% in cells not incubated with rhASA. This background may be explained by a low residual activity of sulfatases in the MLD cells, or some sulfatase activity from the heat-inactivated serum, even though no ASA activity can be detected in the cells or the FCS. This can also explain the low sulfatide metabolism in the control cells in which no rhASA is added.

Experiment	**A**	**B**	**Mean**	**C**	**D**	**Mean**
Added arylsulfatase A (mU/ml)	0	0		25	25	
Metabolised sulfatide (%)	17,3	15,8	16,6	68,7	67,6	68,2
Remaining sulfatide (%)	82,7	84,2	83,4	31,3	32,4	31,8

E	F	Mean	G	H	Mean
50	50		100	100	
69,0	68,7	68,9	68,5	69,1	68,8
31,0	31,3	31,1	31,5	30,9	31,2

Table 3: Degradation of radiolabelled sulfatide in MLD fibroblasts with or without the addition of recombinant human arylsulfatase A (rhASA). The results are given as percent of recovered radioactivity in the cellular lipid fraction.

Time-course experiment:

Experimental design

Cells are loaded with ^{14}C-palmitoyl sulfatide (15 μM) as described above. The medium is changed to medium containing 25 mU/ml affinity-purified rhASA and harvested at 6, 24 and 48 hours. Lipid extracts are prepared and analysed as a described above. The data is expressed as percent of radioactivity of remaining and metabolised sulfatides.

Results

The data from this experiment illustrate that the metabolism of the ^{14}C labelled sulfatide loaded into the MLD fibroblasts increases over 48 hours after addition of affinity-purified rhASA. Data are shown in figure 5.

Conclusion:

From these data it can be concluded that rhASA is efficiently taken up by fibroblasts from a MLD patient and that sulfatides loaded into these fibroblasts can be efficiently metabolised by the exogenous rhASA even at low doses and after incubation for a few hours.

Example 4: Administration of recombinant human rhASA to arylsulphatase A deficient mice.

Effects of rhASA in correcting the sulfatide levels in major target tissues

Materials and methods

rhASA

Purified rhASA was provided by HemeBiotech A/S. The batches of rhASA were G0301 (concentration was 4 mg/ml and the enzyme activity was 166 U/ml) and G0302 (concentration was 4,3 mg/ml and the enzyme activity was 242 U/ml). The rhASA was stored at -20°C. Before start of experiment the two batches of rhASA (G0301 and G0302) were thawed and pooled and the protein content and enzyme activity was analysed. The rhASA in this pool is diluted with TBS so the injection volume was 250-300 µl in all animal groups. The dilutions were made immediately before injection. The body weight and the dose volume were noted for each animal.

Animals

Outbred mice with the mixed genetic background 129/Ola x B6 were used in this study. These mice are descendents from crossings between the male founder mouse (which was heterozygous for the ASA null allele) with wildtype B6 females (Hess et al., 1996). The MLD mice (ASA -/-) are male/females in the age of 20-22 months and with a weight of 25-30 g. The wildtype mice (ASA +/+) are age-matched to the MLD mice.

Blood samples

Blood samples are collected from the tail vein and plasma is immediately prepared and stored at -20°C until analysis. Blood is taken 10 min following iv injection of rhASA.

Termination

The animals were perfused with PBS and sacrificed during or at the end of the studies. Kidneys (both), brain (study b) only), plexus brachialis (both) and nervus ischiaticus (both) are collected and their weights are recorded. The tissues are separately stored at – 20°C until preparation.

ASA ELISA

The amount of rhASA in plasma and tissues were analysed by an ASA specific ELISA. This method is developed by Prof. Gieselmann *et al.* and is routinely used in his laboratory (Matzner et al., 2000). All ELISA determinations are done on individual tissues (no pooling). The brain (study b) only), one kidney, the two ischiatic nerves and the two plexi brachiales of each mouse were homogenized and an aliquot of each homogenate was analysed.

ASA enzymatic activity

The specific enzymatic activity of the rhASA test substance against the synthetic substrate p-Nitrocatecholsulfate (pNCS) was defined. This method is available at Prof. Gieselmanns laboratory (Matzner et al., 2000). No enzyme determinations are done on tissues.

Sulfatide analysis

The sulfatides in tissues were analysed in lipid extracts from tissues by thin layer chromatography (TLC). The method is available at Prof. Gieselmanns laboratory (Matzner et al,. 2002). The analysis was done on individual tissues, with the exception for the N. ischiaticus, which may be pooled within the dose-groups.

Lipids were isolated from Brain, kidney, ischiatic nerve and plexus brachialis by extraction of the 100.000 x g fraction of homogenate aliquots with organic solvents. The extracted total lipids were incubated under alkaline conditions to hydrolyse phospholipids, triglycerides and other lipids which complicate the seperation and detection of the alkaline-resistent lipids sulfatide, sphingomyelin and cholesterol in thin layer chromatography (TLC).
Cholesterol and sphingomyelin, the concentrations of which are unchanged in tissues of ASA knock out mice (Sandhoff et al., 2002; Jan-Eric Mansson, personal communication), served as internal standards and were used to normalize the loading volumes for TLC. TLC plates were processed with $CuSO_4/H_3PO_4$ according to Yao and Rastetter, 1985. To quantify the stained lipids the plates were scanned on a flat bed scanner and the intensities of bands were determined by the analysis program AIDA 2.11 (Raytest, Straubenhard, Germany).

Study a)

Experimental design:

In total 20 male/female MLD mice in the age of 20-22 months were divided into 4 separate groups with five animals/group. Three groups (group 2-4) received different doses of rhASA (10, 20 or 40 mg/kg b.w, respectively) using one iv administration (see Table 1). One group (group 1) was treated with placebo (TBS) and served as negative controls. Five wild type mice (group 5) were included as controls for sulfatide storage. Blood samples were collected from

groups 1-5 10 min after rhASA/placebo administration to verify the injection procedure. The animals were sacrificed 8 days following treatment and the tissue content of sulfatides were analysed in kidney and nervus ischiaticus.

Group	**Animals (number/ type)**	**Dosing**	**Injection volume**	**Time of sacrifice (days after rhASA)**
1	5 MLD	Placebo (TBS)	250-300 µl iv	8 days
2	5 MLD	10 mg/kg rhASA	250-300 µl iv	8 days
3	5 MLD	20 mg/kg rhASA	250-300 µl iv	8 days
4	5 MLD	40 mg/kg rhASA	250-300 µl iv	8 days
5	5 wildtype	No treatment	0	8 days

Table 1: Animal groups where the dosing of rhASA is given as mg/kg body weight (b.w).

Results:

Injection of rhASA was well tolerated and no adverse effects of treatment were visible thereafter.

To control the accurate administration of rhASA into the tail vein, blood samples were taken from all injected animals 10 min after injection and the ASA levels in serum were determined by ELISA. The serum levels were dependent from the injected rhASA dose (Fig. 6) and increased from 0 (0 mg/kg) to 136 (10 mg/kg) to 311 (20 mg/kg) and to 419 µg/ml (40 mg/kg) on average.

Plasma kinetics of rhASA were evaluated after injection of 20 mg/kg rhASA (Fig. 7). As evident from figure 2 serum levels increased to 325 µg/ml five minutes after injection. Over the following 55 minutes the plasma levels were reduced to 113 µg/ml.

Kidney, ischiatic nerve and plexus brachialis show comparable rhASA levels in each dose group. The mean levels range dose-dependently from approximately 0.5 - 1 ng per mg protein (10 mg/kg) to 1.3 - 1.9 ng/mg (20 mg/kg) to 3.8 - 6.1 ng/mg (40 mg/kg) (Fig. 9). In kidney the dependency between dose and mean tissue level is linear. This seems not to be true for the two PNS tissues. Here the doubling of the injected dose from 20 to 40 mg/kg results in an increase of the mean tissue level by a factor of 3 (plexus brachialis) and 5 (nervus ischiaticus), respectively. It has to be mentioned, however, that the amount of PNS tissues was very low so that the determination of protein concentrations and rhASA levels was less precise than for kidney. Therefore, the deviations from linearity might rather reflect methodical limitations of the

assays. This view is supported by the large standard deviations of rhASA levels in plexus brachialis and nervus ischiaticus compared to kidney.

TLC of kidney lipids indicated more than 10-fold higher sulfatide levels in mock-treated ASA knock out mice compared to wildtype controls (Fig. 10A). Upon treatment sulfatide concentrations declined in a manner dependent on the rhASA dosage. Kidneys of the three dose groups showed a reduction of sulfatide by 22% (10 mg/kg), 28% (20 mg/kg) and 43% (40 mg/kg). Thus, approximately half of the sulfatide, which is additionally stored in kidney as a consequence of ASA-deficiency was hydrolysed upon treatment with 40 mg rhASA per kg body weight.

In the plexus brachialis and the ischiatic nerve of ASA-deficient mice sulfatide is approximately 2-fold increased compared to wildtype controls (Fig. 10 B,C). Also in these tissues treatment resulted in a dose-dependent decline of the accumulated sulfatide.

In the plexus brachialis a reduction by 1% (10 mg/kg), 11% (20 mg/kg) and 16% (40 mg/kg) was detectable. Thus, the excess sulfatide was reduced by 29% at the highest rhASA dose.

In the nervus ischiaticus sulfatide was reduced by 10% (10 mg/ml), 16% (20 mg/kg) and 30% (40 mg/kg) upon treatment. This means a reduction of the excess sulfatide by 65% at the highest dose. It is unclear why the ischiatic nerve and plexus brachialis respond differently to substitution therapy with rhASA.

Study b):

General experimental design

In total 20 male/female MLD mice in the age of 20-22 months were divided into 7 separate groups with five animals/group. Five groups (group 2-6) received one dose of rhASA (40 mg/kg b.w) by iv administration (see Table 2). One group (group 1) was treated with placebo (TBS) and served as negative controls. Five wild type mice (group 7) were included as controls for sulfatide storage. Blood samples were collected from groups 1-7 10 minutes after rhASA/placebo administration to verify the injection procedure. The animals were sacrificed 10 minutes, 5 hours, 4 days, 8 days, or 14 days following treatment and the tissue content of sulfatides were analysed in the kidney.

Group	**Animals (number/type)**	**Dosing**	**Injecti n volume**	**Time f sacrifice (minutes/hou rs/days after rhASA)**
1	5 MLD	Placebo (TBS)	250-300 µl iv	10 minutes
2	5 MLD	40 mg/kg rhASA	250-300 µl iv	10 minutes
3	5 MLD	40 mg/kg rhASA	250-300 µl iv	5 hours
4	5 MLD	40 mg/kg rhASA	250-300 µl iv	4 days
5	5 MLD	40 mg/kg rhASA	250-300 µl iv	8 days
6	5 MLD	40 mg/kg rhASA	250-300 µl iv	14 days
7	5 wildtype	No treatment	0	10 minutes

Table 2: Animal groups where the dosing of rhASA is given as mg/kg body

Results:

In the kidney of ASA-deficient mice sulfatide levels decrease approximately two-fold over the course of the study as compared to untreated MLD mice. While sulfatide seen in untreated animals were almost 6-fold higher than those seen in wildtype control mice, the sulphatide levels seen 8 and 16 days after injection of rhASA were only between two and three fold higher compared to wildtype controls (Figure 8)

Study c)

General experimental design:

In total 15 male/female MLD mice in the age of 20-22 months were divided into 5 separate groups with three animals/group. Four groups (group 2-5) received 1, 2, 3 and 4 doses of rhASA, respectively, one dose/week by means of iv administration. The rhASA was injected iv in the tail vein in a volume of approximately 300 µl with a concentration of 20 mg/kg. One group (group 1) is treated with placebo (TBS). Three wild type mice (group 6) were used as controls for sulfatide storage. Blood samples were collected from groups 1-5, 10 min after rhASA administration to verify the injection procedure. The animals were sacrificed one week after receiving the last dose. The MLD mice and wild type mice serving as controls were all sacrificed after four weeks. After the animals were sacrificed the tissues were collected and stored at –20°C, and subsequently the tissue-content of sulfatides was analysed in kidney, brain, plexus brachialis, and nervus ischiaticus.

Group	**Animals (number/type)**	**Dosing**	**Injecti n volume**	**Time of sacrifice (weeks after initiation of study)**
1	3 MLD	Placebo (TBS)	300 µl iv	4 weeks
2	3 MLD	1 x 20 mg/kg rhASA	300 µl iv	1 week
3	3 MLD	2 x 20 mg/kg rhASA	300 µl iv	2 weeks
4	3 MLD	3 x 20 mg/kg rhASA	300 µl iv	3 weeks
5	3 MLD	4 x 20 mg/kg rhASA	300 µl iv	4 weeks
6	3 wildtype	No treatment	0	4 weeks

Table 2: Animal groups where the dosing of rhASA is given as mg/kg body weight (b.w).

Results

In this study TLC of kidney lipids indicated that sulfatide levels were 4.5-fold higher in mock-treated ASA knock out mice compared to wildtype controls (Fig. 11). Whereas a single dose of rhASA did not have any significant effect, sulfatide levels in the kidney were markedly reduced by repeated treatment with the enzyme. Kidneys of the three dose groups receiving 2, 3 and 4 doses showed a reduction of sulfatide by 13%, 29% and 52%, respectively. Thus, approximately 65% of the sulfatide, which is accumulated in kidney as a consequence of ASA-deficiency was hydrolysed upon treatment with one weekly dosis of 20 mg rhASA per kg body weight over 4 weeks.

In the plexus brachialis and the ischiatic nerve of ASA-deficient mice sulfatide is approximately 1.5 to 2-fold increased compared to wildtype controls (Fig. 12 and 13). In the nervus ischiaticus, sulfatide was reduced by 5%, 15%, 24% and 35%, upon treatment for 1, 2, 3 and 4 weeks, respectively. This corresponds to a reduction of the excess sulfatide by 67% at 4 weeks of treatment.

In the plexus brachialis no significant reduction was seen after a single dose of rhASA. However, a reduction by 2%, 11%, and 27% was detectable after 2, 3 and 4 weeks of treatment, respectively. Thus, the excess sulfatide was reduced by 58% at 4 weeks of treatment.

In the brain of ASA-deficient mice sulfatide levels are approximately 1.8-fold increased compared to wildtype controls (Fig. 14), a reduction in sulfatide levels could be seen after 4 weeks of treatment. At this point the total levels of sulfatides were reduced by 13% corresponding to a reduction in excess sulfatides of 30%.

Conclusion

These two studies clearly indicate that intravenous injection of rhASA partially corrects the lipid metabolism of ASA-deficient mice in a dose-dependent manner. Reduction of sulfatide can already be seen at an injection dose of 10 mg/kg in kidney and nervus ischiaticus. In both tissues this dose leads to a tissue level around 1 ng/mg eight days after injection.

The established potential of rhASA to reduce accumulated sulfatide clearly demonstrates that the intravenously injected enzyme is effectively taken up by cells and routed to the lysosomal compartment.

In addition to the positive dose dependency, the studies have further shown that correction of the pathophysiological levels of sulfatide resulting from the deficiency in Arylsulfatase A activity increases markedly with treatment over 4 weeks. This unexpected high therapeutic potential of treatment over an extended period of time indicates that long-term treatment of individuals suffering from Metachromatic Leukodystrophy may result in sulfatide levels approaching those seen in unaffected individuals. All tissues except brain demonstrate an almost linear decline in sulfatide levels. This is in further support of the probability that the entire bulk of sulfatide is accesssible for rhASA.

Reduction of sulfatide in the nervous system is expected to result in the amelioration of neurologic deficits, which develop progressively in ASA-deficient mice and human patients. Thus, a reduction of storage material in peripheral nerves might result in an improvement of the gait pattern, general motor activity and rotor rod performance described for ASA-deficient mice at an age around 1 year (Hess et al., 1996).

Of most concern, however, are the severe symptoms relating to the accumulation of sulfatides in the central nervous system. In light hereof, the observed 30% reduction in sulfatide levels in the brain upon 4 weeks of treatment is extremely interesting. In addition, the observation of a significant effect of rhASA in the brain upon simple intravenous administration is highly surprising since it is generally believed that the use of specific delivery systems are required in order for peptides to gain access across the blood-brain barrier. Efficient delivery systems with a broad applicability are currently not available and the ability of rhASA in itself to elicit an effect in the brain is therefore a promising new aspect relating to its use.

Example 5: Rotarod studies

General experimental design:

Motor learning tasks were performed using an accelerating rotarod apparatus. This equipment is based on a rotating cylinder covered with texture rubber. The apparatus is divided into multiple sections allowing several mice to be tested simultaneously, one per section. Mice walk forward on the rotating cylinder during the four-minute test sessions and their performance is evaluated on the basis of the number of mice remainin on the rod at the end of the session. Optionally the speed of the rotating can be increased during the session, for instance from 4 to 40 rpm.

In the present experiments, two groups of mice are tested: Group 1 consists of mice which are from 8 to 10 months old and group 2 consists of mice which are from 10 to 12 months old. All mice except wild type controls received three doses of either placebo (Tris buffered saline) or rhASA (20 mg/kg body weight). Rotarod studies were performed before treatment and one month after the first dose was given.

Results:

Data from the rotarod studies are presented in figure 15, however, only data from studies on group 2 are presented. The data clearly illustrate a beneficial effect of the rhASA treatment on motor learning skills one month after initiation of the treatment. Before treatment, 43% of the wildtype mice but only 18% of the micee destined for placebo treatment and 10 % of the mice destined for rhASA treatment were able to stay on the rod during the entire session. One month after initiation of treatment, 65% of the wildtype mice, 13% of the placebo treated mice and 31% of the rhASA treated mice were successful. When comparing results from rotarod experiments on groups 1 and 2, a significant deterioration of the performance is seen with increasing age. Thus, only 25% of the older mice were successful. Still, however, their performance was increased by the rhASA treatment indicating that rhASA has positive effects also in a late disease stage.

Example 6: Studies on nerve motor conduction velocity

General experimental design:

Mice were anaesthetized and placed on a heating pad or under a heating lamp in order to avoid hypothermia. The mice were further secured in order to prevent movement artifacts due to the electrical stimulation and the lower limbs were gently stretched in order to facilitate the measurements of distances between proximal and distal points of stimulation. The motor conduction velocity of the sciatic nerve (MCV) was obtained by stimulating the nerve with steel monopolar needle electrodes. A pair of stimulating electrodes was inserted subcutaneously near

the nerve at the ankle; a second pair of electrodes were placed at the isciatic notch, in order to obtain two distinct sites of stimulation, distal and proximal, respectively, along the nerve. The muscular response to the electrical nerve stimulation, the compound motor action potential (cAMP) was recorded with a pair of recording needle electrodes; the active electrode was inserted into muscles in the middle of the paw, while the reference was placed in the skin between the first and the second digit. Motor evoked potentials (MEP) were recorded with the same montage described for MVC for the paw muscle with transcranial electrical stimulation of the motor cortex. The excitatory volleys descending from the cortico-spinal pathways evoke a motor potential in the paw muscles through a transsynaptic depolarization of alfa-motoneuron (cortical MEP, cMEP). The peripheral conduction time(PCT) was obtained using a method based on the F-wave latency determination. In fact, the antodromic volley following nerve stimulation excites the alfa-motoneuron, giving rise to spikes travelling ortodromically up to the muscle, where they can be recorded as late potential F-Wave. The PCT was thus calculated with the formula ($F\text{-}wave_{lat}+cAMP_{lat}-1)/2$. The central conduction time (CCT) was measured as the difference between cAMP and PCT latencies. An unpared Student's *t*-test was performed for statistical evaluation of the data.

Results:

Determination of electrophysiological parameters

raw data

	distal mot lat	*durati on*	*ampl distal*	*f-wave*	*prox lat*	*dur prox*	*ampl prox*	*distanc e*	*nerve cond vel*
wild-type number									
43	0,84	3	19,7	4,88	1,48	2,9	17,9	24	37,50
44	1	3,6	21,6	5,72	1,6	2,9	19,7	24	40,00
40	0,96	2,9	*	5,48	1,52	3,2	*	26	46,43
37	0,88	3,7	17,4	6,04	1,6	3,2	15,8	26	36,11
45	0,68	2,9	19,8	5,2	1,36	3	16,5	26	38,24
39	0,76	3,2	19	5,68	1,44	3,2	15,9	25	36,76
38	0,76	3,8	16,3	6	1,4	3,3	13,8	25	39,06
mean	**0,84**	**3,30**	**17,40**	**5,57**	**1,49**	**3,10**	**15,41**	**25,14**	**39,16**
SD	**0,11**	**0,36**	**4,15**	**0,39**	**0,09**	**0,15**	**3,37**	**0,83**	**3,21**

ko TBS

number									
6	0,76	3,9	10,3	5,68	1,36	3,1	6,9	25	41,67
4	0,84	4	11,3	5,64	1,44	3,3	11,7	24	40,00
2	1	4,3	12,4	5,32	1,4	nd	13,6	27	67,50
3	0,8	4,8	15,7	5,64	1,44	3,6	14,8	25	39,06
7	0,76	4	16,8	4,76	1,32	nd	11,7	25	44,64
1	0,84	4,2	17	5,48	1,36	nd	12,8	25	48,08
5	0,8	4	23	5,08	1,4	nd	18,1	26	43,33
8	0,68	3,7	18,1	5,04	1,32	nd	16,2	25	39,06
mean	**0,81**	**4,11**	**15,58**	**5,33**	**1,38**	**3,33**	**13,23**	**25,25**	**45,42**
SD	**0,09**	**0,31**	**3,89**	**0,32**	**0,04**	**0,21**	**3,16**	**0,83**	**8,83**

ko									
rhASA									
number									
26	0,76	4,3	28,5	5,2	1,36	nd	24	25	41,67
11	0,84	4,3	25,3	5,88	1,36	nd	22,6	24	46,15
27	0,68	4	12,7	5,08	1,36	nd	12,9	26	38,24
18	0,8	3,6	22,2	5,16	1,32	nd	17,2	25	48,08
14	0,72	3,4	17,7	4,88	1,32	nd	17,1	26	43,33
30	0,68	3,5	16,8	4,68	1,24	nd	14,3	25	44,64
24	0,76	3,6	12,7	5	1,32	nd	11,4	27	48,21
25	0,84	3,5	27,5	5	1,44	nd	11,9	24	40,00
mean	**0,76**	**3,78**	**20,43**	**5,11**	**1,34**	**nd**	**16,43**	**25,25**	**43,79**
SD	**0,06**	**0,35**	**5,94**	**0,33**	**0,05**	**nd**	**4,46**	**0,97**	**3,43**

* wildtype mouse #40 yielded low amplitudes due to technical problems (ampl dist = 8.0; ampl prox = 8.3); nd - duration after proximal stimulation not determinable

5

statistical evaluation using Student's t-test

P values

	dml	duration	ampl distal	f-wave	prox lat	dur prox	ampl prox	distance	NLG
wildtype untreated vs knockout mock-treated	0,2930	**0,0004**	**0,0449**	0,1203	**0,0073**	nd	**0,0258**	nd	0,0611
knockout mock-treated vs knockout rhASA-treated	0,1149	**0,0375**	**0,0461**	0,1121	0,0745	nd	0,0720	nd	0,3282

grey - statistically significant difference ($P < 0.05$)

Results

- the following changes in the electrophysiological pattern of knockout mice are statistically significant (wildtype vs mock-treated knockouts):
  - duration (of amplitude after distal stimulation) is increased
  - amplitude (height) after distal stimulation is decreased
  - latency after proximal stimulation is decreased
  - amplitude (height) after proximal stimulation is decreased

- treatment results in the following statistically significant changes of the pattern (mock-treated knockouts vs rhASA-treated knockouts)
  - duration (of amplitude after distal stimulation) is decreased towards normal values
  - amplitude (height) after distal stimulation is increased towards normal values

(1) The amplitude is the result (sum) of individual axon potentials. If all axon potentials pass the recording electrode at the same time point the amplitude would be short and high. If the potentials pass it at different time points (because some axons conduct fast and others slow) the amplitude would be broad and low. Compared to wildtype mice the amplitude of ASA knockout mice is more flattened and extended.

(2) For the determination of the nerve conductance velocity the time between stimulation and begin of the amplitude is measured. This time is virtually the same for knockout and wildtype mice. It can be concluded that the knockout mice possess nerve fibers with normal conductance velocity.

From (1) and (2) it can be concluded that knockout mice have fast conducting fibers (normal nerve conductance velocity), but also a substantial fraction of fibers which conductance velocity is more or less reduced (flattened and extended amplitude).

Treatment results in a significant improvement of the duration and height of the amplitude. Thus, rhASA seems to increase the conductance velocity of the blocked fibers so that they conduct more similar to the fast fibers.

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CLAIMS

1. A process for production of rhASA in a continuous cell culture system, the process comprising:

i) culturing a mammalian cell capable of producing rhASA in liquid medium in a system comprising one or more bio-reactors;

ii) concentrating, purifying and formulating the rhASA by a purification process comprising one or more steps of affinity chromatography.

2. A process according to claim 1, wherein said mammalian cell produces a glycoform of rhASA, which are efficiently endocytosed *in vivo* via the mannose-6-phosphate receptor pathway.

3. A process according to claim 2, wherein said one or more glycoforms of rhASA comprises an amount of exposed mannose-6-phosphate, which allows efficient endocytosis of rhASA *in vivo* via the mannose-6-phosphate pathway.

4. A process according to claims 2 or 3, wherein at least one of said one or more glycoforms of rhASA are similar to a glycoform produced in CHO cells.

5. A process according to any of the preceding claims wherein the mammalian cells are of human or primate origin.

6. A process according to any of the preceding claims, wherein the mammalian cells are CHO cells.

7. A process according to claim 6, wherein said CHO cells are CHO-DG44 cells

8. A process according to any of the preceding claims, wherein said cells comprise a nucleic acid sequence which encodes an amino acid sequence according to SEQ ID NO: 2.

9. A process according to any of the preceding claims, wherein said rhASA is encoded by SEQ ID NO: 1.

10. A process according to any of the preceding claims, wherein said cells are propagated in a serum-free medium, which serum-free medium only contains recombinant human proteins that have molecular weights less than 10 Kd in a concentration of less than 100 ug/ml.

11. A process according to any of the preceding claims, wherein the production phase extends for at least two weeks and wherein 1 to 2 reactor volumes of cell culture are harvested each day.

12. A process according to any of the preceding claims, wherein said on or more bio-reactors have a volume of 1 L or more.

13. A process according to any of the preceding claims, wherein said bio-reactor(s) are equipped with cell retention devices and re-circulation loops.

14. A process according to any of the preceding claims, wherein the concentration and purification process of ii) comprises the following steps:

I) concentrating rhASA present in the liquid medium by tangential flow filtration;

II) loading the rhASA containing supernatant obtained in step I on an equilibrated chromatography column and eluting one or more fraction(s) containing rhASA;

III) loading the fraction(s) from step II on another equilibrated chromatography column and eluting one or more fraction(s) containing rhASA;

IV) purifying rhASA present in the fraction(s) from step III by tangential flow filtration;

V) polishing the preparation of rhASA from step IV in one or two or more successive steps, each step comprising loading the preparation on an equilibrated chromatography columns and eluting one or more fraction(s) containing rhASA;

VI) passing the fraction(s) from step V through a viral reduction filter;

VII) formulating the fraction(s) from step VI in order to obtain a preparation of rhASA in a suitable formulation buffer;

VIII) optionally filling the formulated preparation of rhASA into a suitable container and freeze-drying the sample.

15. A process according to claim 14, wherein the chromatography column used in step II of the purification process is an anion exchange column.

16. A process according to claim 15, wherein said anion exchange column is a DEAE Sepharose column.

17. A process according to any of claims 14 to 16, wherein the chromatography column used in step III of the purification process is a hydrophobic interaction column.

18. A process according to claim 17, wherein said hydrophobic interaction column is a butyl sepharose column or a phenyl sepharose column.

19. A process according to any of claims 14 to 18, wherein steps II and III of the purification process are performed in the reverse order.

20. A process according to any of claims 14 to 19, wherein purification of the sample in step IV of the purification process is accomplished by tangential flow filtration.

21. A process according to claim 20, wherein said tangential flow filtration occurs against a Biomax A-screen.

22. A process according to any of claims 14 to 21, wherein the two or more successive steps in step V of the purification process comprise a passive step, wherein the rhASA passes through an affinity chromatography resin or membrane, and an active step, wherein the rhASA is detained within and subsequently eluted from an anion exchange membrane or resin.

23. A process according to claim 22,wherein said anion exchange membrane or resin is a high resolving anion exchanger.

24. A process according to claim 22, wherein said affinity chromatography chromatography membrane or resin and said anion exchange membrane or resin are coupled in a series.

25. A process according to any of claims 22 to 24, wherein said affinity chromatography chromatography membrane or resin is a Mustang™ S membrane or a Blue Sepharose resin and said anion exchange membrane or resin is a Mustang™Q membrane or Resource™ Q resin.

26. A process according to any of claims 14 to 25, wherein the filtration of the sample as performed in step VI of the purification process is replaced by contacting the sample with a detergent prior to step I of the purification process.

27. A process according to any of the preceding claims which results in a product comprising a relative amount of bioactive rhASA which is at least 98% of the total amount of proteins as determined by reverse phase HPLC.

28. A process according to any of the preceding claims wherein the rhASA is formulated in an isotonic solution such as 0.9% NaCl and 10-50 mM Sodium phosphate pH 6.5 - 8.0 or sodium phosphate, glycine, mannitol or the corresponding potassium salts.

29. A process according to any of claims 1 to 28, wherein the rhASA is formulated in a physiological buffer, such as:

a) formulation buffer I containing (in mM): Na_2HPO_4 (3.50 - 3.90), NaH_2PO_4 (0 - 0.5), Glycine (25 - 30), Mannitol (230 - 270), and water for injection; or

b) formulation buffer II containing (in mM): Tris-HCl (10), Glycine (25 - 30), Mannitol (230 - 270), and water for injection.

30. A process according to any of the preceding claims wherein the rhASA is formulated as lipid vesicles comprising galactoside and/or phosphatidyl choline and/or phosphatidyl ethanolamine.

31. A process according to any of the preceding claims wherein the rhASA is formulated as a sustained release formulation involving bio-degradable microspheres, such as microspheres comprising polylactic acid, polyglycolic acid or mixtures of these.

32. A process according to any of claims 1-27 and 30-31 wherein the rhASA is formulated with a hypertonic solution in order to cause osmotic opening of the blood-brain barrier.

33. A process according to any of the preceding claims wherein the rhASA is formulated in a solution comprising an enhancer for nasal administration.

34. A process according to any of the preceding claims wherein the rhASA is formulated so as to enhance its half-life in the bloodstream and/or reduce clearing via the kidneys and/or prevent extended uptake via the liver.

35. A process according to any of the preceding claims resulting in production, purification and formulation of a protein, which is enzymatically equivalent to rhASA.

36. A pharmaceutical composition comprising rhASA, which is efficiently endocytosed via the mannose-6-phosphate receptor pathway in vivo.

37. A pharmaceutical composition according to claim 36 wherein said composition comprises at least 98% bioactive rhASA as determined by reverse phase HPLC.

38. A pharmaceutical composition according to claim 36 or 37, wherein said rhASA has a specific activity of at least 25 u/mg.

39. A pharmaceutical composition according to any of claims 36 to 38, wherein said composition comprises a peptide or polypeptide capable of facilitating the entry of rhASA into the central nervous system.

40. A pharmaceutical composition according to claims 36 to 38, wherein said composition does not comprise a peptide or polypeptide capable of facilitating the entry of rhASA into the central nervous system.

41. A pharmaceutical composition according to any of claims 36 to 40, wherein said composition comprises intact cells.

42. A pharmaceutical composition according to any of claims 36 to 40, wherein said composition does not comprise intact cells.

43. A pharmaceutical composition according to any of claims 36 to 42, wherein said rhASA is obtainable by the process according to any of claims 1-35.

44. A rhASA, according to any of claims 1 to 35 as a medicament.

45. A rhASA according to claim 44 as a medicament for reducing the galactosyl sulphatide levels within target cells in the peripheral nervous system and/or within the central nervous system in a subject.

46. A rhASA according to claims 44 or 45 as a medicament, the administration of which leads to decreased impairment of motor-learning skills.

47. A rhASA according to any of claims 44 to 46 as a medicament, the administration of which leads to increased nerve motor conduction velocity and/or nerve conduction amplitude.

48. Use of a rhASA for the manufacture of a medicament for reducing the galactosyl sulphatide levels within target cells in the peripheral nervous system and/or within the central nervous system in a subject.

49. Use according to claim 48, wherein said rhASA is obtainable by a process according to any of claims 1 - 35

50. A method of treating a subject in need thereof, said method comprising administering to said subject a pharmaceutical composition comprising a rhASA according to any of claims 1 - 35 and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within said subject.

51. A method according to claim 50, said method comprising administering said pharmaceutical composition intravenously to said subject and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within the peripheral nervous system in said subject.

52. A method according to claims 50 and 51, said method comprising administering said pharmaceutical composition intravenously and by spinal injection and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within the peripheral nervous system and in target cells within the central nervous system in said subject.

53. A method according to any of claims 50 to 52, said method comprising administering said pharmaceutical composition intravenously to said subject and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within the peripheral nervous system and in target cells within the central nervous system in said subject.

54. A method according to any of claims 50 to 53, wherein rhASA is efficiently endocytosed in vivo into target cells within a tissue selected from the group comprising liver, kidney, spleen, heart.

55. A method according to any of claims 50 to 53, wherein said target cells within the central nervous system are oligodendroglia.

56. A method according to any of claims 50 to 53, wherein said target cells within the peripheral nervous system are Schwann cells.

57. A method according to any of claims 50 to 56, wherein said pharmaceutical composition is administered in one or more doses, each dose comprising an amount of rhASA which is within the range of 0.1 to 100 mg/kg body weight.

58. A method according to any of claims 50 to 57, wherein said pharmaceutical composition is administered on a daily, weekly, bi-weekly or monthly basis.

59. A method according to any of claims 50 to 58, wherein intravenous and or spinal injection of said pharmaceutical composition is performed as a supplement to bone marrow transplantation.

60. A method according to any of claims 50 to 59, wherein said reduction in the galactosyl sulphatide levels in target cells within the central nervous system is due at least in part to a washout effect.

61. A method according to claim 50 to 60, wherein said pharmaceutical composition further comprises a hypertonic solution or wherein said pharmaceutical composition is administered together with a hypertonic solution in order to cause osmotic opening of the blood-brain barrier.

ABSTRACT

The present invention pertains to a process for production of recombinant human arylsulfatase A in a cell culture system, the process comprising culturing a mammalian cell capable of producing rhASA in liquid medium in a system comprising one or more bio-reactors; and concentrating, purifying and formulating the rhASA by a purification process comprising one or more steps of affinity chromatography. Other aspects of the invention provides a pharmaceutical composition comprising rhASA, which is efficiently endocytosed via the mannose-6-phosphate receptor pathway *in vivo* as well as a rhASA a medicament and use of a rhASA for the manufacture of a medicament for reducing the galactosyl sulphatide levels within target cells in the peripheral nervous system and/or within the central nervous system in a subject. A final aspect of the invention provides a method of treating a subject in need thereof, said method comprising administering to said subject a pharmaceutical composition comprising a rhASA and thereby obtaining a reduction in the galactosyl sulphatide levels in target cells within said subject.

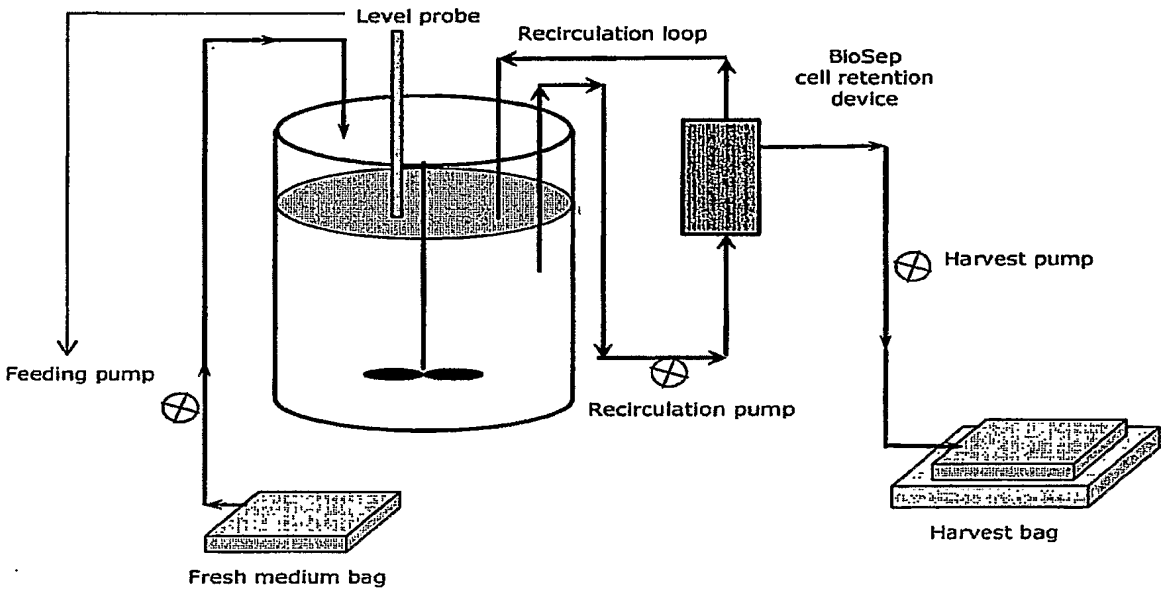


Fig.1

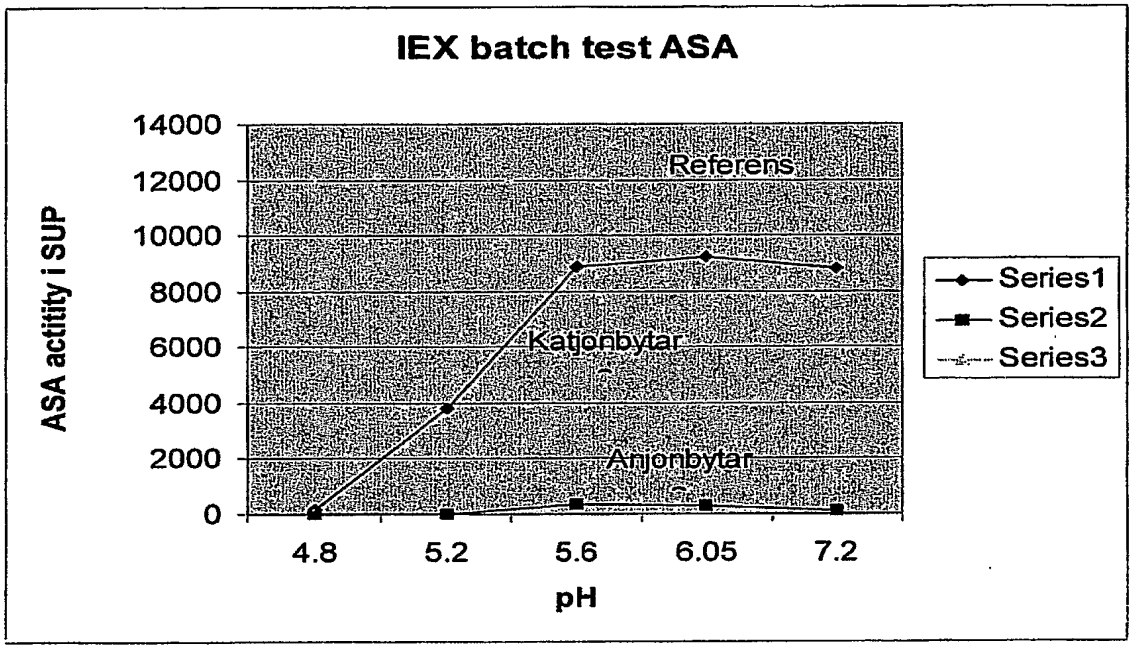
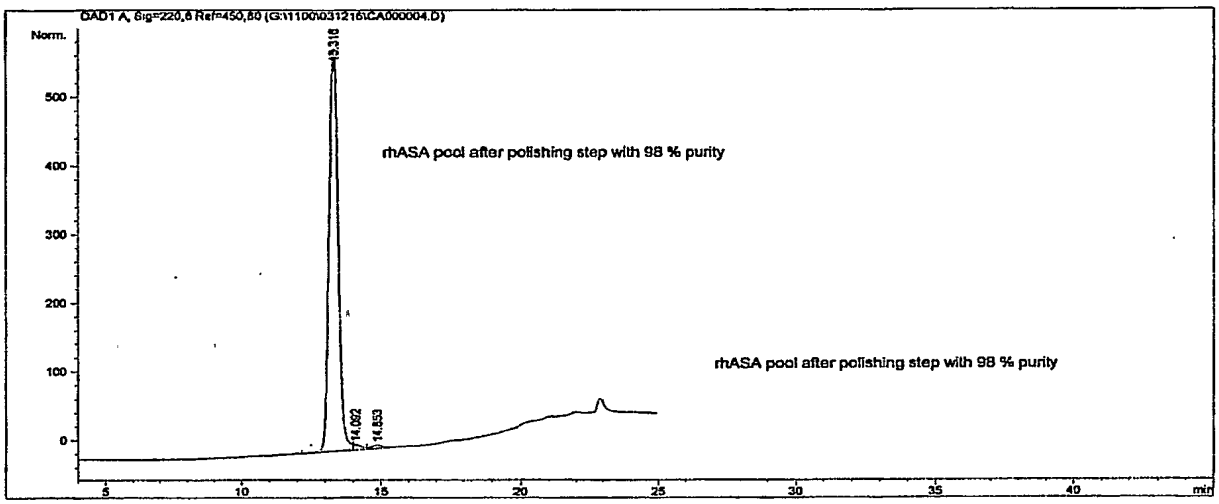


Fig.2

A



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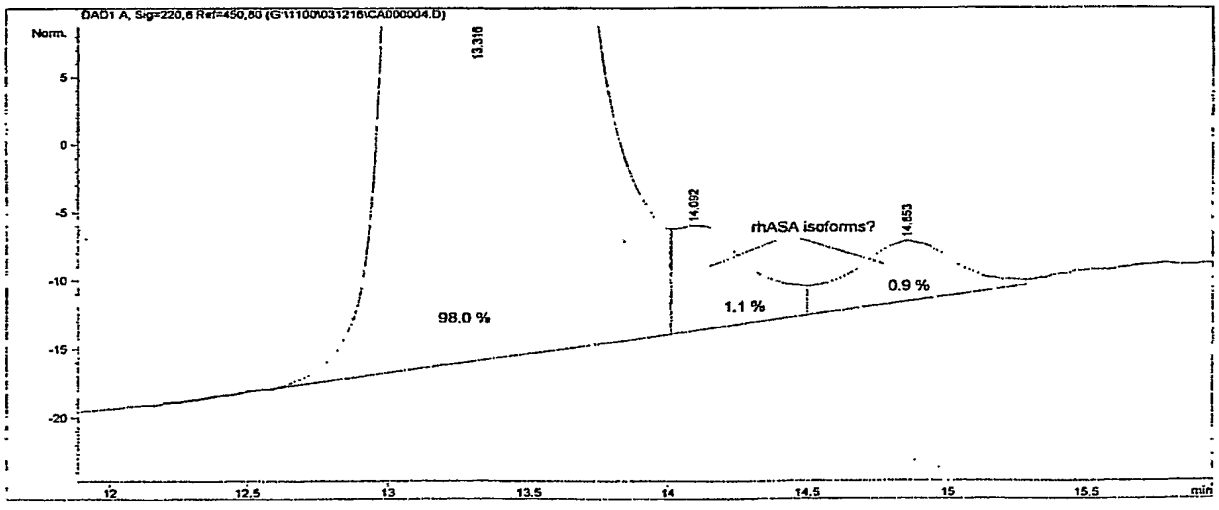


Fig.3

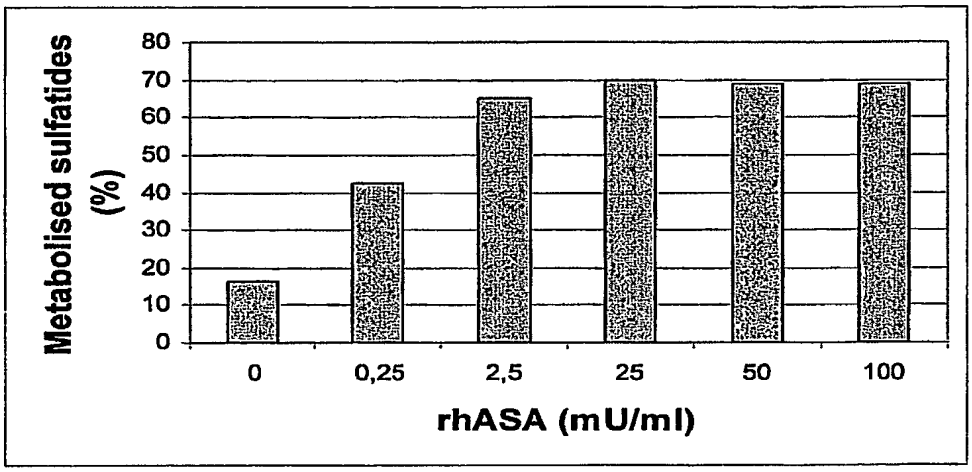


Fig.4

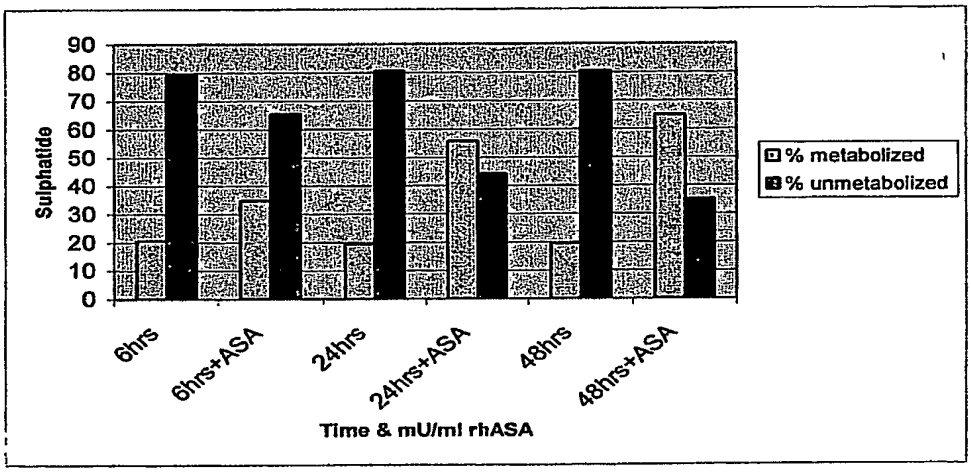


Fig.5

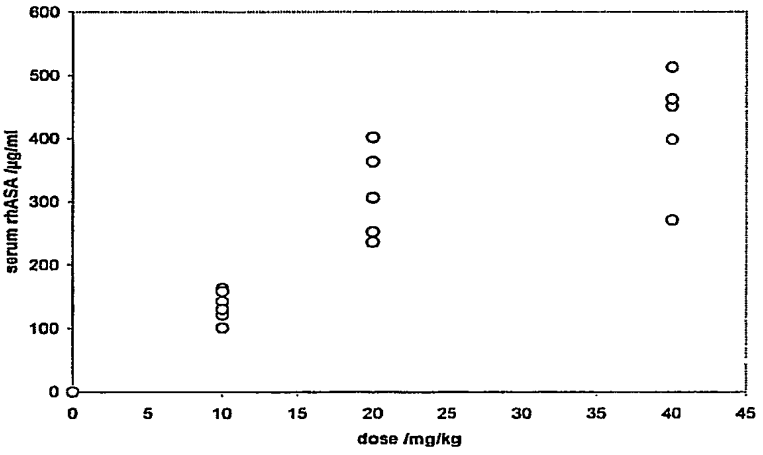


Fig.6

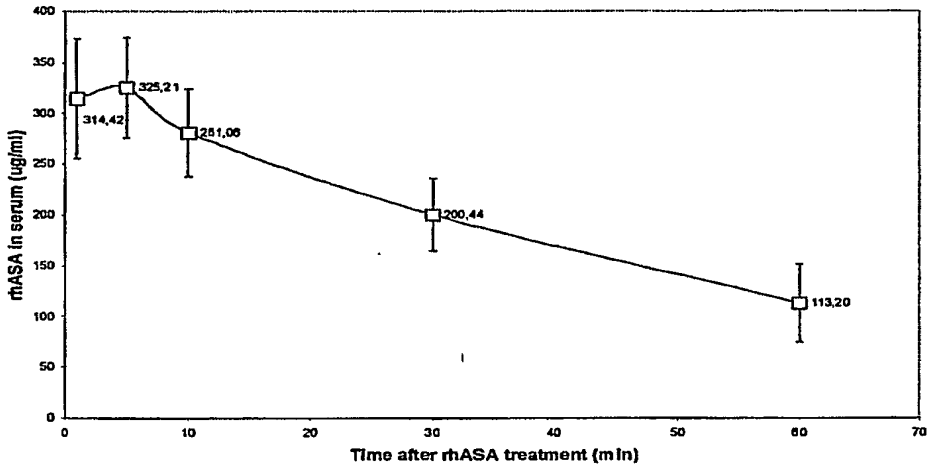


Fig. 7

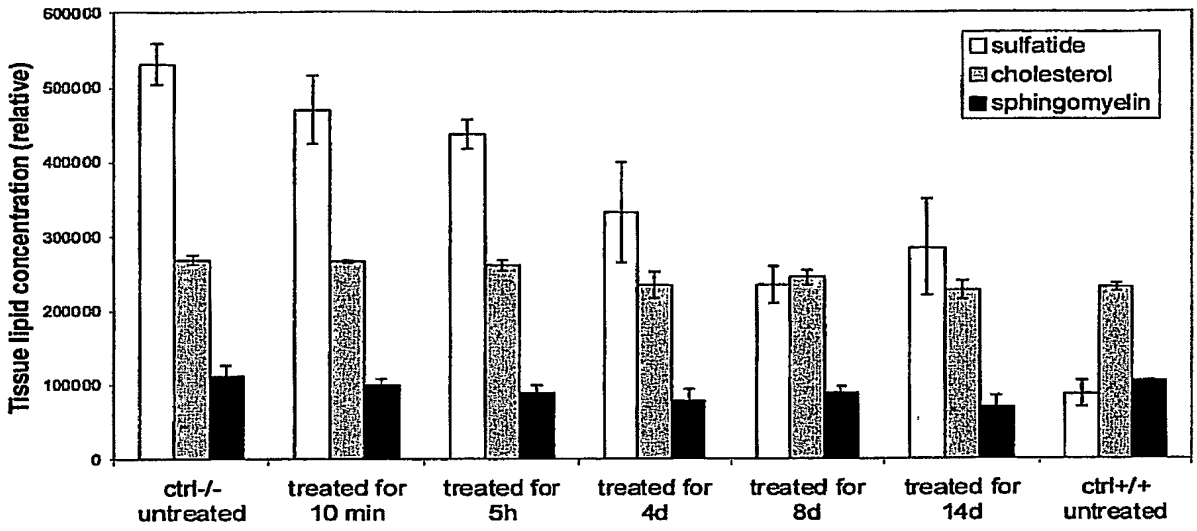
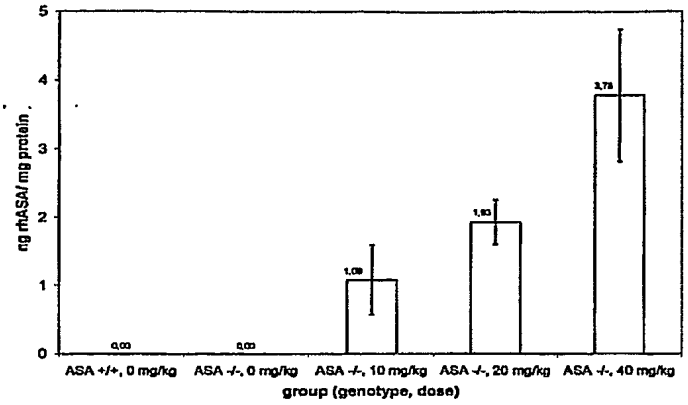


Fig. 8

A



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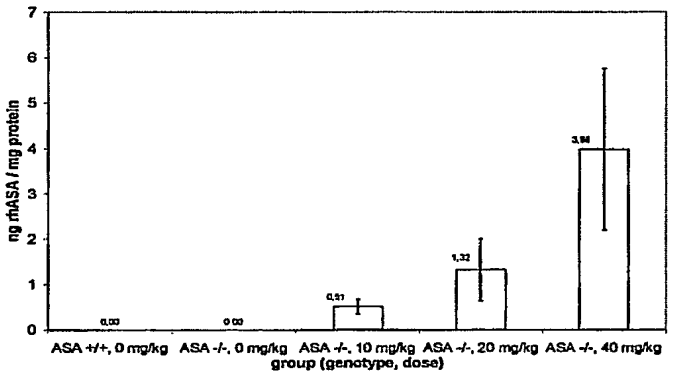


C

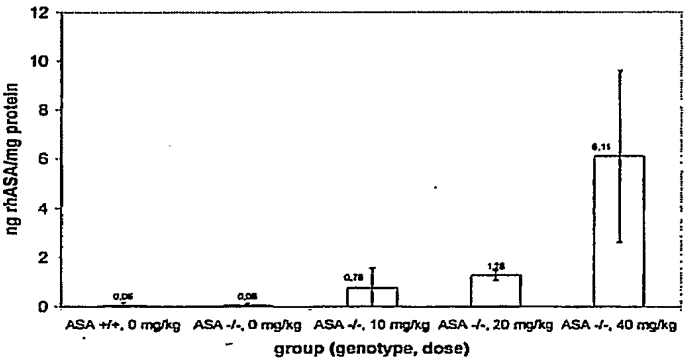
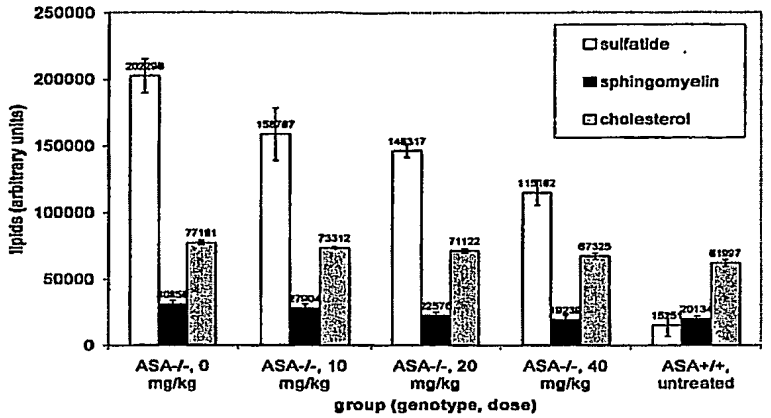
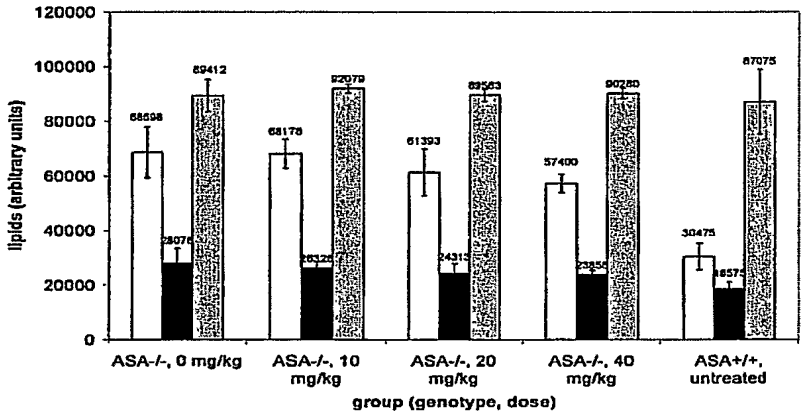


Fig. 9

A



B



C

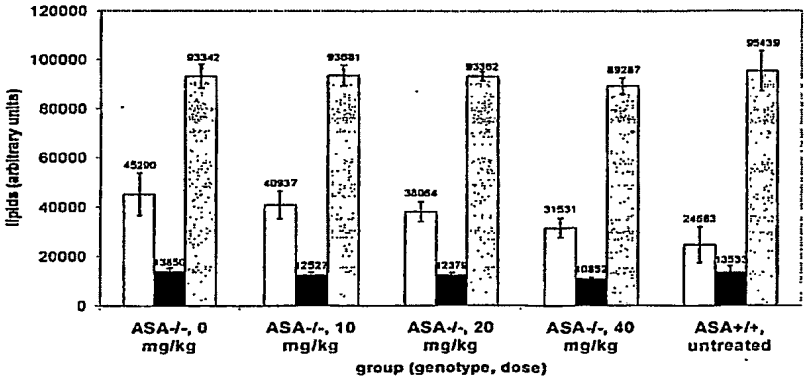


Fig. 10

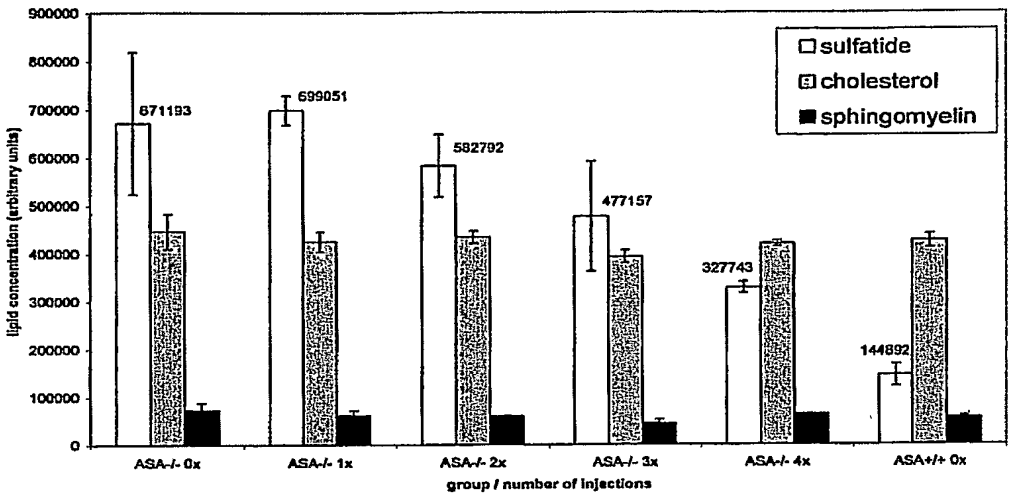


Fig.11

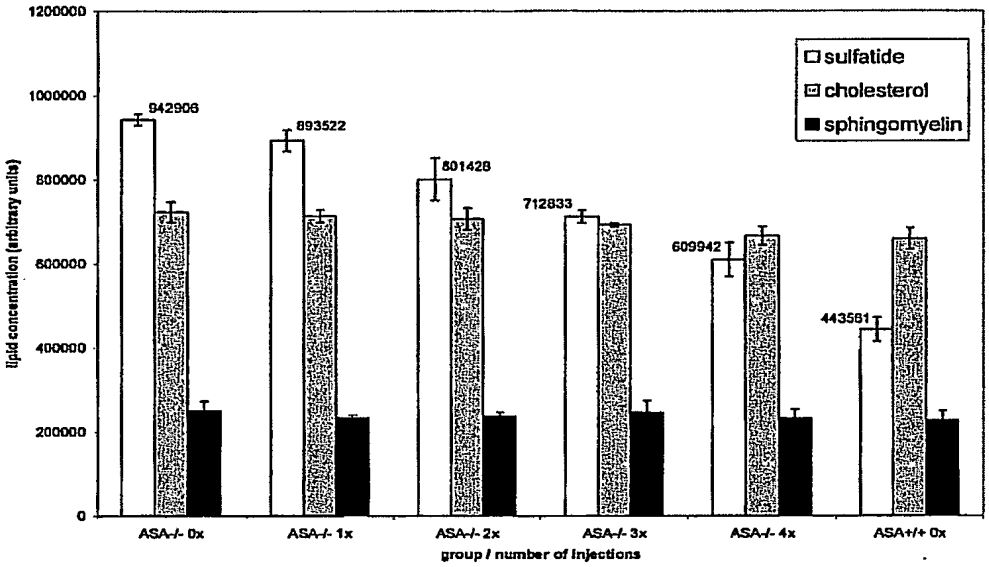


Fig.12

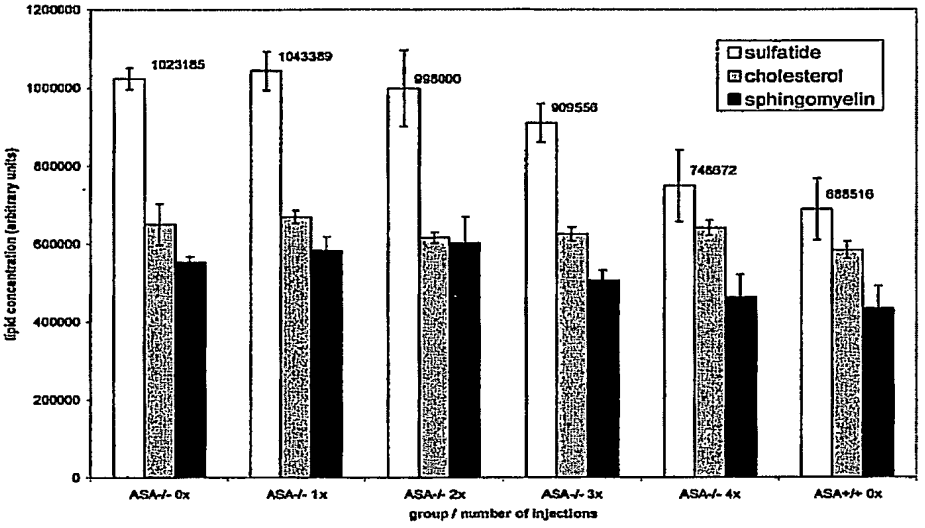


Fig. 13

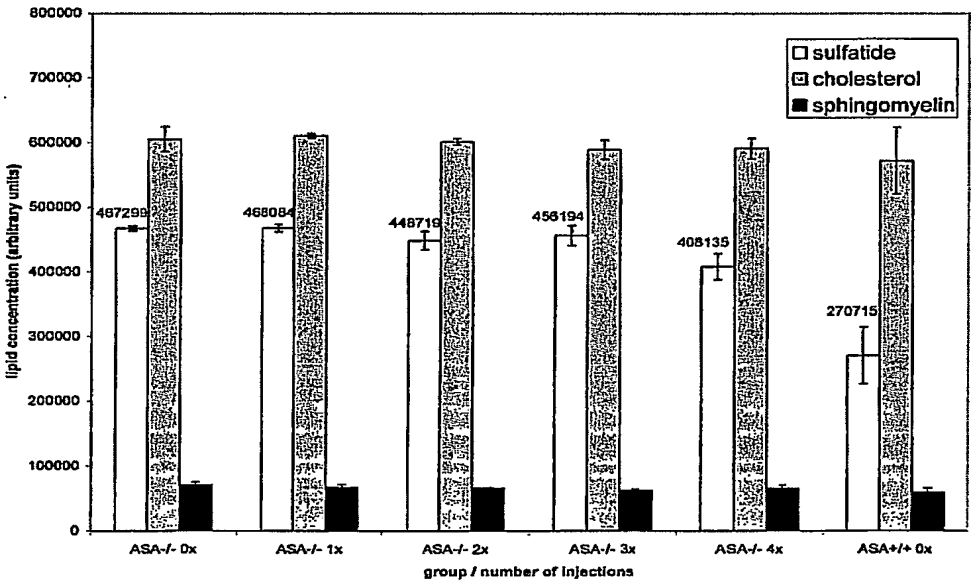
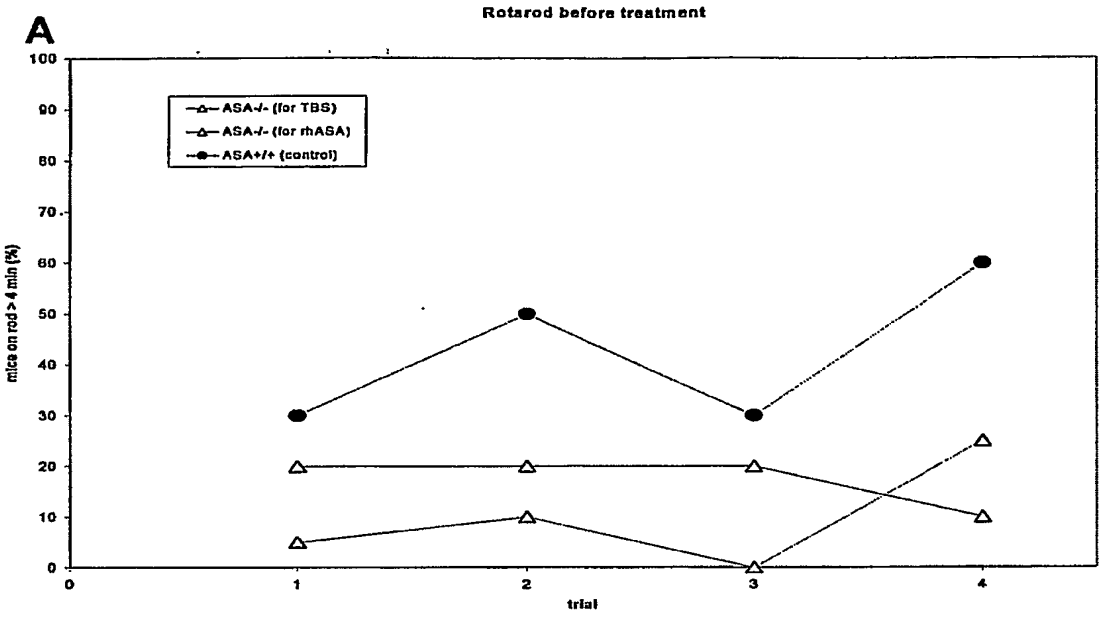


Fig.14



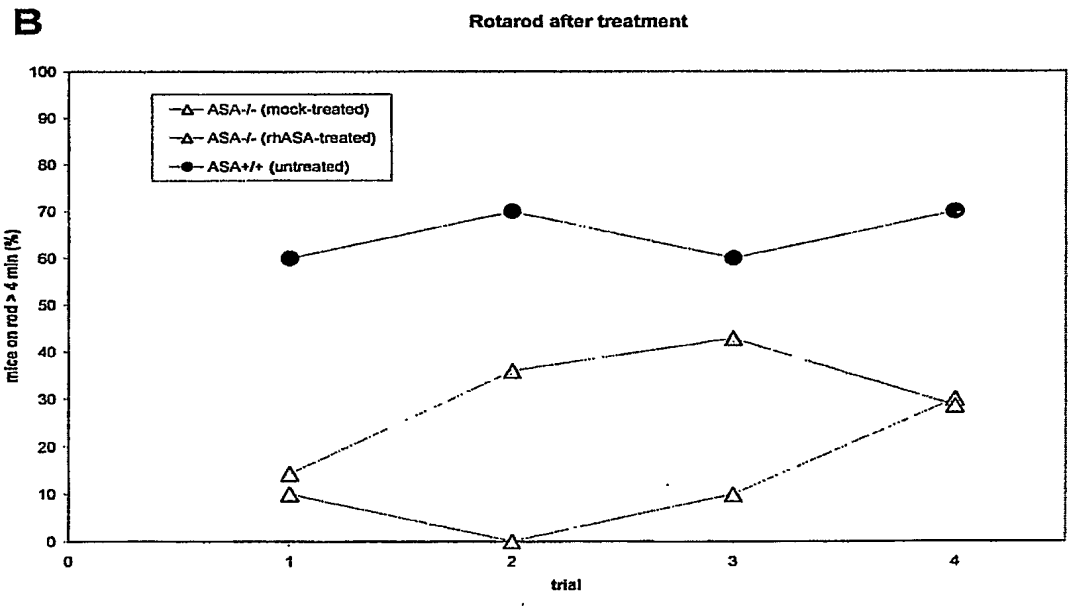


Fig.15